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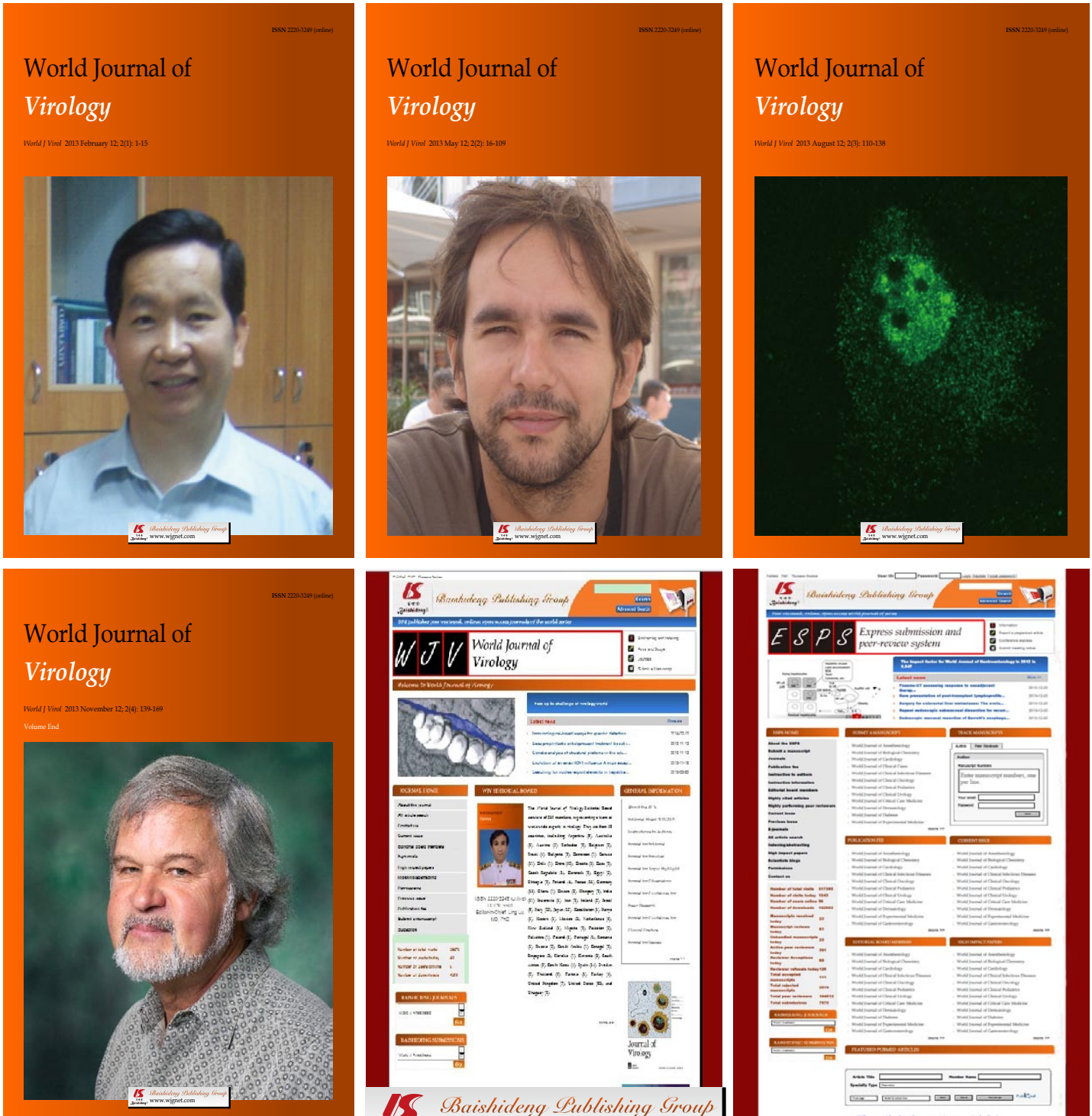

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EDITORIAL

Betanodavirus: Mitochondrial disruption and necrotic cell death

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Abstract

Betanodaviruses cause viral nervous necrosis, an infectious neuropathological condition in fish that is characterized by necrosis of the central nervous system, including the brain and retina. This disease can cause mass mortality in larval and juvenile populations of several teleost species and is of global economic importance. The mechanism of brain and retina damage during betanodavirus infection is poorly understood. In this review, we will focus recent results that highlight betanodavirus infection-induced molecular death mechanisms *in vitro*. Betanodavirus can induce host cellular death and post-apoptotic necrosis in fish cells. Betanodavirus-induced necrotic cell death is also correlated with loss of mitochondrial membrane potential in fish cells, as this necrotic cell death is blocked by the mitochondrial membrane permeability transition pore inhibitor bongkrekic acid and the expression of the anti-apoptotic Bcl-2 family member zfBcl-xL. Moreover, this mitochondria-mediated necrotic cell death may require a caspase-independent pathway. A possible cellular death pathway involving mitochondrial function and the modulator zfBcl-xs is discussed which may provide new insights into the necrotic pathogenesis of betanodavirus.

Key words: Nervous necrosis virus; Mitochondrial membrane potential; Bongkrekic acid; zfBcl-xL; Caspase-independent; Brain damage

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BETANODAVIRUS

Betanodaviruses cause viral nervous necrosis, an infectious neuropathological condition in fish that is characterized by necrosis of the central nervous system, including the brain and retina[1]. This disease can cause mass mortality in larval and juvenile populations of several teleost species and is of global economic importance[2].

The family *Nodaviridae* is comprised of the genera *Alphanodavirus* and *Betanodavirus*. *Alphanodavirus* predominantly infects insects, while *Betanodavirus* predominantly infects fish[3-7]. Nodaviruses are small, nonenveloped, spherical viruses with bipartite positive-sense RNA genomes (RNA1 and RNA2) that are capped but not polyadenylated[3]. RNA1 encodes a non-structural protein of approximately 110 kDa that has been designated RNA-dependent RNA polymerase or protein A. This protein is vital for replication of the viral genome. RNA2 encodes a 42 kDa capsid protein that may also function in the induction of cell death[8,9]. Nodaviruses also synthesize RNA3, a sub-genomic RNA species from the 3' terminus of RNA1. RNA3 contains two putative open reading frames that potentially encode a 111 amino-acid protein B1 and a 75 amino-acid protein B2[3,10,11]. Recently, the betanodavirus B1 protein has been shown to have an anti-necrotic death function during the early replication stages[10]. In contrast, the betanodavirus B2 protein appears to function as a suppressor of host siRNA silencing[12,13] or as a

necrotic death factor[14,15]. In addition, red-spotted grouper nervous necrosis virus (RGNNV) infection and expression can trigger the ER stress response, which results in the upregulation of the 78 kDa glucose regulated protein at the early replication stage[16]. Very recently, RGNNV has been shown to induce the production of reactive oxygen species (ROS) during the early and middle replication stages[17].

NECROTIC CELL DEATH DURING BETANODAVIRUS INFECTION

Apoptosis and necrosis are two stereotyped mechanisms by which nucleated eukaryotic cells die[18,19]. Necrosis is considered a pathological reaction to major perturbations in the cellular environment such as anoxia[20], while apoptosis is a physiological process that preserves homeostasis by facilitating normal tissue turnover[21,22]. The mechanisms leading to apoptosis are better understood[23-26].

Tumor necrosis factor-α (TNF-α) is a crucial regulator of the innate and adaptive immune response against microbial infection *via* its regulation of cell death and survival[27]. TNF-α is a pro-inflammatory cytokine that plays important roles in diverse host responses, such as cell proliferation, differentiation, necrosis, apoptosis, and induction of other cytokines. Recently, TNF-α has been shown to induce either nuclear factor κB-initiated survival or apoptosis, depending on the cellular context[28]. As such, many viruses have strategies to neutralize TNF-α either by direct binding and inhibition of the ligand or receptor or by modulation of various downstream signaling events[29,30].

The death receptors (DRs), including TNF receptor-1 (TNF-R1), Fas, DR3, DR4, DR5, and TRAIL, contain an intracellular "death domain" that influences downstream signaling pathways by means of homotypic interactions with adaptor proteins, such as FADD, TRADD, and receptor-interacting protein-1 (RIP1)[31]. These DRs induce apoptosis in many cell types through activation of caspase 8. Activated caspase 8 may act indirectly to induce apoptosis through cleavage of Bid. The truncated Bid protein acts on the mitochondria to cause the release of cytochrome c, which further activates downstream caspase 9. Furthermore, TNF-R1 is also involved in the initiation of necrotic cell death (Figure 1)[32]. TNFα and other cytokines that bind to receptors of different-classes promote the generation of ROS, which functions as a second messenger in the necrotic cell death pathway[33,34].

RIP1 is an intracellular adaptor molecule with kinase activity[35]. The RIP1[36] and RIP3[37] proteins appear to be crucial for the initiation of caspase-independent cell death. RIP1 is also necessary for the generation of ROS by TNF-α[33,34].

Other research has shown that TNF-α activates RIP1 kinase-mediated signaling, promoting the induction of downstream genes influencing necrosis or apoptosis[38].

In aquatic betanodavirus systems, RGNNV induces exposure of phosphatidylserine (PS; an early apoptotic marker) at an early apoptotic stage[39], as determined by annexin-V assays. Secondary necrotic morphological changes are also evident at middle and late stages under phase-contrast microscopy in RGNNV-infected grouper cells using acridine orange (AO) and ethidium bromide (EtBr) to identify apoptotic and post-apoptotic necrotic cells; double-stained cells are often observed. Furthermore, RGNNV infection can induce ROS production in mitochondria at the early replication stage [24 h postinfection (*p.i.*)]. Viral expression during this stage leads to ROS production, triggering an oxidative stress response[17], which may contribute to secondary necrotic cell death. In our system, RGNNV induces necrotic cell death, but whether or not this requires RIP1 kinase-mediated signaling is still unknown.

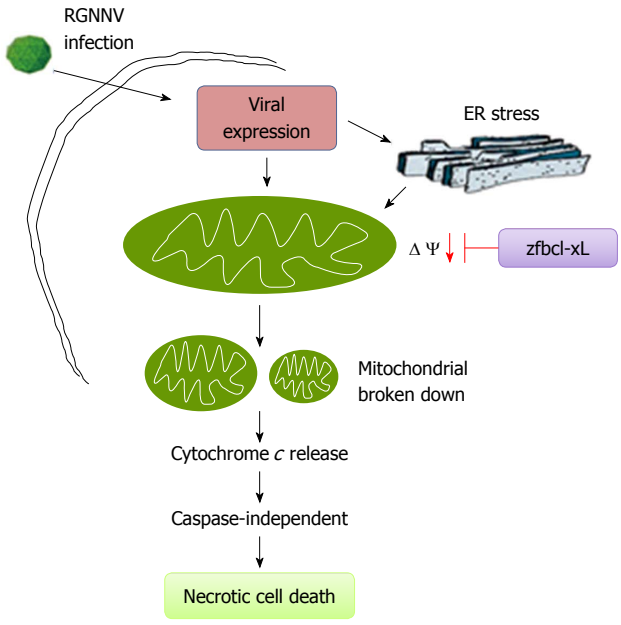

Figure 1 Schematic working model for mitochondrial dysfunction caused by betanodavirus infection. Red-spotted grouper nervous necrosis virus infection and early replication causes an ER stress response upon entry in the early replication stage [12 h postinfection (*p.i.*)]. Subsequently, this ER stress signaling can affect a number of important events, including enhanced viral expression, induction of mitochondrial membrane potential (MMP) loss, mitochondrial breakdown, and cytochrome c release at the middle replication stage (48 h *p.i.*). Bcl-2 may also be downregulated at the middle replication stage, as overexpression of Bcl-xL prevents loss of MMP. In regard to downstream events, cytochrome c release is not required or caspases activation or triggering of necrotic cell death at the late replication stage (72 h *p.i.*).

BETANODAVIRUS INFECTION AFFECTS MITOCHONDRIAL FUNCTION

Apoptosis is controlled at the mitochondrial level by the sequestration of apoptogenic proteins in the mitochondrial intermembrane space and the cytosolic release of these factors on exposure to proapoptotic signals[39,40]. Disruption of the mitochondrial membrane potential (MMP) initiates the caspase cascade, leading to downstream activation of apoptosis[40,41]. MMP can affect both the inner and outer mitochondrial membranes, and this

precedes the signs of necrotic or apoptotic cell death, including the apoptosis-specific activation of caspases[42]. Adenine nucleotide translocase (ANT) plays a role in the exchange of ATP for ADP through the inner mitochondrial membrane, thus supplying the cytoplasm with ATP newly synthesized by oxidative phosphorylation. In a search for proapoptotic proteins, Bauer *et al*[43] identified the protein ANT1 as the main inducer of apoptosis. The overexpression of ANT1 produces rapid cell death, with a concomitant decrease in MMP and an increase in nucleosomal DNA degradation. Since this cell death is sensitive to caspase inhibitors and to inhibitors of the mitochondrial permeability transition pore (MPTP), such as bongkrekic acid (BKA), apoptosis and the involvement of MPTP are thus implicated[43]. Hence, the mitochondrion is appreciated as a central integrator of pro-death stimuli, streamlining various types of proapoptotic signals into a common caspase-dependent pathway[41].

In a betanodavirus system, secondary necrosis is correlated with loss of MMP in grouper liver cells[44] and mitochondrial breakdown at the middle and late apoptotic stages[11]. The loss of MMP is dramatically inhibited by the ANT specific inhibitor BKA, which enhances host-cell viability at the early and middle apoptotic stages[44]. Furthermore, RGNNV-induced mitochondrial cytochrome c release is also blocked following BKA treatment at the early (24 h *p.i.*) and middle (48 h *p.i.*) stages.

THE ROLE OF ANTI-APOPTOTIC BCL-2 FAMILY MEMBERS DURING BETANODAVIRUS INFECTION

Apoptosis removes damaged, infected, and superfluous cells. In most circumstances, a cell's decision to live or die rests largely with the Bcl-2 family of interacting proteins[45,46]. The Bcl-2 family of proteins includes both anti- and pro-apoptotic molecules that act at a critical intracellular decision point along a common death pathway[47]. The ratio of antagonists (Bcl-2, Bcl- xL, Mcl-1, Bcl-W, and A1) to agonists (Bax, Bak, Bcl-xS, Bid, Bik, Bad, PUMA, and NOXA) dictates whether a cell responds to a proximal apoptotic stimulus[46,47]. The Bcl-2 family member proteins also interact with mitochondria to regulate MMP[42]. Changes in MMP, which can include permeabilization of both the inner and outer membranes, precede necrotic or apoptotic cell death[40], highlighting the central role of the mitochondrion as a integrator of pro-death stimuli[41]. Cytochrome *c* release from mitochondria into the cytosol is initiated by the interaction of mitochondria with one or more members of the Bcl-2 family. Thus, Bcl-2 proteins, which critically regulate apoptosis, function prior to the irreversible damage of cellular constituents[48-50].

In our fish system, we found that RGNNV infection can induce downregulation of the anti-apoptotic Bcl-2 genes at the middle apoptotic stage (48 h *p.i.*)[16]. Subsequently, mitochondrial damage and RGNNV-induced necrotic cell death were assessed in stable cell lines producing the anti-apoptotic Bcl-2 proteins, zfBcl-xL or zfMcl-1a. Both zfBcl-xL and zfMcl-1a strongly inhibited RGNNV-induced necrotic cell death and reduced the percentage of necrotic cells at 36 h *p.i.* by up to 90% (zfBcl-xL) and 93% (zfMcl-1a), respectively, when compared with the NNV-infected control group. Cell viability was correspondingly enhanced at 36 h *p.i.* by 102% (zfBcl-xL) and 98% (zfMcl-1a), respectively, when compared with the NNV-infected control group[11]. Furthermore, overexpression of zfBcl-xL dramatically blocked RGNNV viral death factor protein α[9] and B2[14] induction of cell death.

CASPASE-INDEPENDENT DEATH PATHWAY IN BETANODAVIRUS-INFECTED CELLS

The mitochondrion is seen as a central integrator of pro-death stimuli, streamlining various types of proapoptic signals into a common caspase-dependent pathway[41], although the absolute requirement for caspase activation in apoptosis is no longer considered dogma[51,52].

The molecular cornerstones of apoptosis are the family of cysteinyl aspartate-specific proteases, collectively known as caspases. At least 13 caspases have been identified[53], and members of this family can be subdivided into two groups: initiators and executioners. Initiator caspases serve to relay death signals from proapoptotic signals to executioner caspases, which then cleave key proteins involved in cellular structure and function. Known initiators include caspase 8 and caspase 9, whereas known effectors include caspase 3[54], caspase 6, and caspase 7.

Our analysis of caspase 3, caspase 8, and caspase 9 activities revealed no significant differences relative to normal control cells at 0, 24, 48, and 72 h *p.i.* with RGNNV (MOI = 5), and cell death was not effectively blocked by treatment with a pan-caspase inhibitor[11]. The results of these assays suggest that betanodavirus can induce caspase-dependent and caspase-independent death pathways that may be dependent on the specific cell line used. In grouper liver cells, RGNNV may preferentially induce caspase-independent death, but GGNNV induces caspase-dependent death in sea bass cells[8].

CONCLUSION

We have reviewed the cellular impact of RGNN viral infection on cell viability *via* modulation of mitochondrial necrotic cell death in fish cells. Over recent years, our knowledge about mitochondria-mediated apoptotic cell death has expanded, but our understanding of mitochondria-mediated necrotic cell death is still limited, especially in lower vertebrates. In addition, we are beginning to uncover the physiological roles of mitochondria-mediated caspase-independent necrotic cell death. However, despite these recent advances, many questions remain largely unanswered. What signaling occurs upstream of

necrotic cell death following betanodavirus infection? Does induction of autophagy affect necrotic cell death during viral replication? What parameters, in addition to mitochondria-shaping proteins, control mitochondrial fusion and fission[15,55]? Hopefully, future studies will increase our understanding of the mechanisms underlying mitochondria-mediated necrosis, its functions in multiple biological processes, and the regulatory signaling pathways that control its activation. This knowledge will be of great importance for validating mitochondria-mediated necrosis as an effective target for the treatment of various diseases, including RNA viral infections.

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MINIREVIEW

Hepatitis C virus resistance to new specifically-targeted antiviral therapy: A public health perspective

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Abstract

Until very recently, treatment for chronic hepatitis C virus (HCV) infection has been based on the combination of two non-viral specific drugs: pegylated interferon-α and ribavirin, which is effective in, overall, about 40%-50% of cases. To improve the response to treatment, novel drugs have been designed to specifically block viral proteins. Multiple compounds are under development, and the approval for clinical use of the first of such direct-acting antivirals in 2011 (Telaprevir and Boceprevir), represents a milestone in HCV treatment. HCV therapeutics is entering a new expanding era, and a highly-effective cure is envisioned for the first time since the discovery of the virus in 1989. However, any antiviral treatment may be limited by the capacity of the virus to overcome the selective pressure of new drugs, generating antiviral resistance. Here, we try to provide a basic overview of new treatments, HCV resistance to new antivirals and some considerations derived from a Public Health perspective, using HCV resistance to protease and polymerase inhibitors as examples.

Key words: Specifically-targeted antiviral therapy; Direct-acting antiviral; Protease inhibitors; Polymerase inhibitors; Viral resistance

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INTRODUCTION

Hepatitis C virus (HCV) infects an estimated 170 million people worldwide, which represents around 2%-3% of the global population[1]. Chronic HCV infection causes a progressive liver disease associated with increased risk of liver cirrhosis and hepatocellular carcinoma[2]. When end-stage liver disease is established, the only reliable therapeutic intervention, liver transplantation, is limited by the fact that a new chronic hepatitis is established in the graft, which can be lost in the early years post-transplantation[3]. The burden of HCV disease varies throughout the world, with country-specific prevalence ranging from < 1% to > 10%. The epidemiology of HCV infection is changing, and the transmission routes, demographics of infected individuals, and HCV genotype distribution varies between countries[4,5]. Public Health policies will likely need

to be adapted to these differences. With the new availability of highly effective therapies, there is now a time of increasing opportunities to significantly reduce HCV-related morbidity and mortality. Until very recently the standard treatment for chronic HCV infection was the combination of pegylated interferon-α (Peg-IFNα) and ribavirin (RBV)[6]. Rather than targeting the virus directly, these drugs are immunomodulators, although RBV may also increase the mutation rate of the HCV genome[7]. The majority of responder patients remain virus-free after 5 years of follow-up and are considered to be cured[8]. However, the efficacy of this therapy is variable, ranging from 40% to 80% depending on: viral genotype, stage of liver fibrosis, viral load, side effects and treatment discontinuation, body-mass index, age, race, and host genetics[9]. Genome-wide association studies revealed single nucleotide polymorphisms in the promoter region of the *IL28B* gene, encoding interferon lambda-3, as the strongest predictors for treatment response[10]. Increasing response rates are expected due to the development of numerous new direct-acting antivirals (DAAs) active against HCV (STAT-C: Specifically-Targeted Antiviral-Treatment for hepatitis C). Some of these compounds are in advanced clinical trials to be used either as an adjunct to Peg-IFNα + RBV, and/or combined with other DAAs. STAT-C drugs include inhibitors of the viral proteins NS3/4A, NS4B, NS5A, and NS5B[11,12]. The well-defined virus-specific enzymatic functions of the NS3/4A serine protease and the NS5B RNA-dependent RNA-polymerase (RdRp) made them the initial focus for drug development and represent the most advanced STAT-C drugs, showing potent antiviral efficacy *in vitro* and *in vivo*. In 2011, the NS3/4A protease inhibitors (PIs) Telaprevir (Vertex Pharmaceuticals) and Boceprevir (Schering-Merck) became the first STAT-C compounds approved for clinical use; and NS5A inhibitors and several NS5B polymerase inhibitors entered phase Ⅱ of development[11]. In addition, STAT-C drugs targeting other HCV proteins and drugs directed to host proteins that interact with the virus have also entered clinical development. There is a glimpse of optimism in the field, hoping that new STAT-C medications will allow shorter treatment durations and increase the rates of patients responding to antiviral treatment[13].

From a Public Health perspective, the extension of new, more effective, treatments including STAT-C compounds will: (1) eventually reduce the disease burden of chronic hepatitis C in the near future; (2) reduce the long-term costs of delayed care by increasing efforts to screen undiagnosed cases, with the aim of giving access to treatment and preventing progression of the disease; and (3) require a reinforcement of Public Health surveillance[14].

However, this optimism may be tempered by evidence demonstrating that HCV variants resistant to STAT-C compounds are rapidly selected *in vitro* and *in vivo*[15-17]. Eventually all classes of STAT-C, including NS3/4A protease and NS5B polymerase inhibitors, select for HCV resistant variants, although some nucleosidic inhibitors exhibit a higher barrier to resistance[18]. This is not surprising, given the high error rate of HCV replication and the rapid turnover of circulating virions. In fact, resistant variants arise from preexisting subpopulations of viral genomes already circulating in the infected individual, before therapy is started[19]. Selective drug pressure changes the balance between the different intra-individual HCV quasispecies; and resistant genomes dominate the circulating viruses in patients with treatment failure or suboptimal treatment response, as evidenced in patients treated with NS3/4 PIs[20-22]. Fortunately, resistant variants remain sensitive to Peg-IFN + RBV, which still makes their elimination possible with the current Peg-IFN + RBV treatment[16]. However, these therapy regimens are complex, and simplification is eagerly pursued. Finally, most of the first-generation STAT-C compounds have been designed using HCV proteins and replicon assays based in HCV subtype 1b, and the efficacy in other viral subtypes may be suboptimal (lower genetic barrier), given the diversity of this virus and the high number of genotypes and subtypes[23]. Due to the low genetic barrier (the number of nucleotide substitutions required for the virus to acquire resistance to a given drug) of some compounds, there are concerns that high-level resistance will develop quickly, and the possibility of transmission of resistant strains among intravenous drug users[24]. From a Public Health perspective, it seems necessary: (1) to determine the prevalence of major HCV resistant variants in the infected population; (2) to determine the efficacy of STAT-C compounds on HCV subtypes other than 1b; and (3) to establish virology laboratories for HCV genotypic resistance testing and surveillance. Finally, because the distribution of HCV subtypes varies in different geographical regions, policies may have to be refined locally to give access to treatment with optimized STAT-C regimes.

HCV BIOLOGY AND THE BASIS FOR RESISTANCE

HCV is an enveloped virus, the only member of the genus *Hepacivirus* within the family *Flaviviridae*, with a positive-sense, single-stranded RNA genome of about 9600 bases flanked by two highly-conserved non-coding regions[25]. The genome encodes a single polyprotein of around 3000 amino acids, processed by both host and viral proteases into the mature three structural and seven non-structural proteins, including the components of the capsid and envelope (core, E1 and E2) and the viral enzymes needed for replication and virion assembly (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B), respectively (Figure 1)[26]. Like other RNA viruses, the high error rate of the RdRp makes HCV evolution very rapid, with a mutation rate estimated at 10^{-3} to 10^{-5} nucleotides per site[27].

Evolution of the virus has led to the distinction of six major genotypes and more than 40 subtypes. Genotypes 1, 2 and 3 are more common in Western countries, genotypes 1, 4 and 5 in Africa, and genotype 6 in Asia[23]. The distribution of HCV genotypes and subtypes varies be-

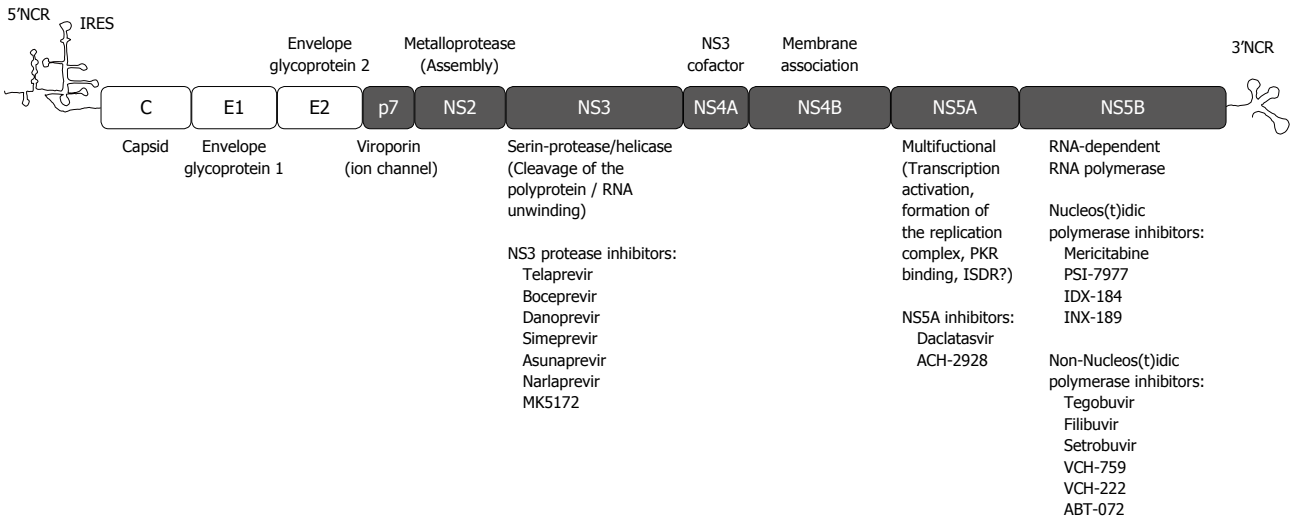

Figure 1 Diagram of the hepatitis C virus genes and the viral polyprotein, with two non-coding regions in the 5' and 3' ends of the viral genome, structural (with) and non-structural (grey) proteins. The targets for the most-developed Specifically-Targeted Antiviral-Treatment for hepatitis C compounds are indicated, together with drugs in advanced development.

tween countries probably because of the spread through different routes of transmission, such as blood-transfusion and healthcare-related practices (subtype 1b) or intravenous drug use (subtype 1a and genotype 3) which is now the main route of infection in most countries[4]. For instance, the prevalence of HCV subtype 1b varies from more than 50% of infected individuals in Italy, Poland, Romania, Turkey, or Russia to less than 25% in Canada. Similarly, the prevalence of non-1 genotypes is also very variable, accounting from less than 25% of the infections in Romania, Turkey, or the Czech Republic to more than 50% in Sweden, Norway, or the United Kingdom[4]. Local HCV genotype/subtype epidemiology may be relevant to the design of optimal treatment strategies because the cross-genotype efficacy of most STAT-C compounds is very limited (see below).

HCV displays another level of genetic variability: intraindividual variation. The combination of a high mutation rate with the production of around 10^{12} virions per day[28] results in every infected individual carrying a pool of slightly variant viral genomes which can eventually contain every possible single (and maybe double) mutants: a cloud of variants common in RNA viruses, so-called "quasispecies"[21,29].

This extensive genetic variability gives HCV the capacity to generate drug resistance. Mutations that change amino acids of STAT-C target proteins, including NS3 and NS5B, do occur in the absence of the drugs, and can cause conformational changes that may interfere with drug-target interaction. If DAA-resistant variants are already present before the start of treatment, they will be rapidly selected and become dominant in the viral quasispecies once the drug is administered, because they will be under positive selective pressure. In addition, unless the replication of the virus is fully suppressed, even if resistance is not present prior to therapy, adaptive mutations can eventually emerge during STAT-C administration, that reduce the susceptibility of the virus to the drug.

Not surprisingly, several mutations were soon identified *in vitro* to be associated with reduced susceptibility to NS3 and NS5B inhibitors[17], some of these are present in natural isolates from naive individuals[30-35], and were later related to STAT-C treatment failure in clinical trials[18].

The role of naturally-occurring variations in resistance to STAT-C inhibitors is therefore a focus of intensive research. An example is the study of HCV resistance to NS3/4A protease and NS5B polymerase inhibitors. Table 1 shows a summary, extracted from several reviews, of the most important amino acid variations in HCV NS3/4A protease, NS5A protein and NS5B polymerase associated with resistance to DAAs.

HCV resistance to NS3/4A PIs

The viral *NS3* gene encodes amino acids 1027-1657 of the polyprotein (numbering on HCV-H77-1a strain), including a serine protease located in the N-terminal domain (amino acids NS3 1-181) and an NTPase/RNA helicase in the C-terminal part (amino acids NS3 182-623)[36]. The chymotrypsin-like protease requires a cofactor, the NS4A protein, and is responsible for critical steps in the virus lifecycle: (1) the cleavage of the viral polyprotein in the NS3-NS4A, NS4A-NS4B, NS4B-NS5A and NS5A-NS5B junctions; and (2) the modification of the cellular response, interfering with the interferon pathway[37-39]. Thus, blocking NS3/4A protease activity may inhibit both processing of the viral polyprotein and viral down-regulation of the innate immune response.

Clinical development of the first-in-class HCV PI (Ciluprevir, BILN-2061) showed exceptional antiviral activity both *in vitro* and *in vivo* in phase I Studies, but further development was halted because cardiotoxicity was detected in animals[40]. These promising results were reproduced with other PIs, such as Telaprevir (VX-950), Boceprevir

Table 1 Most important variations related to hepatitis C virus viral resistance to NS3 protease and NS5b polymerase *in vitro* and/or *in vivo*

Inhibitor class	Amino acid variations implicated in resistance
First generation NS3/A PI	
Ciluprevir	(NS3) R155K/T/Q, A156V/T, D168A/V/T/H
Telaprevir	(NS3) V36M/A, T54A, R155K/T, A156V/T/S, V36M/A+R155K/T, V36M/A+A156V/T
Boceprevir	(NS3) V36M/A/L, T54S/A, R155K, V55A, R155T, A156S, V158I, V170A, I170T
Second generation NS3/A PI	
Danoprevir	(NS3) R155K, D168E
Simeprevir	(NS3) Q80R/Q, R155K/T/Q, A156S/V/T, D168A/V/T/H
Asunaprevir	(NS3) R155K, A156V/T, D168A/E/T/V/Y
Narlaprevir	(NS3) V36A/M, R155K/T/Q, A156S/V/T, V170A
MK5172	(NS3) A156V/T, D168A/V/T/H
NS5A inhibitors	
Daclatasvir	(NS5A) Q30R, L31 M/V, Y93C/N
NA polymerase inhibitors	
Mericitabine	(NS5B) S282T *in vitro*, not reported *in vivo*
PSI-7977	Not reported *in vivo*
IDX-184	Not reported *in vivo*
INX-189	Not reported
NNI polymerase inhibitors	
Tegobuvir	(NS5B) C316N, Y448H
Filibuvir	(NS5B) M423T/I/V, M426T, I482T
Setrobuvir	(NS5B) M414T/L, G554D, D559G
VCH-759	(NS5B) L419M/V, M423T/I/V, I482L/V/T, V494I/A
VCH-222	(NS5B) L419M, M423T, I482L
ABT-072	(NS5B) C316Y, M414T, Y448H/C, S556G

Summary from[11,17,18,77-80]. PI: Protease inhibitors; NA: Nucleos(t)ide analogs; NNI: Non-nucleos(t)idic (allosteric) inhibitors.

(SCH-503034), and Danoprevir (ITMN-191). Several other PIs belonging to two inhibitor classes are in clinical trials: linear ketoamids, and macrocyclic compounds, including Simeprevir (TMC435, Tibotec/Medivir), Asunaprevir (BMS-650032, Bristol-Myers Squibb), Danoprevir (RG7227, Roche/InterMune), BI201335 (Boehringer-Ingelheim), ACH-1625 and ACH-2684 (Achillion), Vaniprevir and MK-5172 (Merck and Co.)[11,12].

Two of these have been approved recently for clinical use: Telaprevir and Boceprevir. HCV resistance to both linear and macrocyclic PIs by amino acid substitutions is well documented *in vitro* and *in vivo*[41]. The selection of resistant mutants is rapid (during the first weeks of exposure to the DAA), and compound-specific, although some resistant strains however may show a reduced fitness, which allows viral control using the standard Peg-IFN + RBV treatment[16]. A major concern of HCV resistance to first-generation PIs is cross-resistance. Substitutions NS3-R155K/T and NS3-A156S/T/V confer a high level of resistance to both Boceprevir and Telaprevir and cross-resistance to most NS3 PIs. Substitutions NS3-V36A/M and NS3-T54A/S confer a low level of resistance to both Telaprevir and Boceprevir, and NS3-V170A/T to Boceprevir. There is also some cross-resistance of mutations in positions 36, 54 and 170 with other compounds, while changes in positions NS3-80, NS3-155 and NS3-168 are implicated in resistance to macrocyclic inhibitors[17,42]. Double mutants may also be selected by STAT-C treatments, combining two resistance mutations for the same or different PI class, with a potential for broad resistance to both linear and macrocyclic inhibitors[43]. However, given the available experience with human immunodeficiency virus, the selection of double or triple HCV mutants resistant to different drugs targeting different viral genes (*i.e.*, protease and polymerase) seems unlikely. Selected resistant variants in the protease have been implicated in late relapse after cessation of treatment, and may decline or remain detectable for years after treatment failure[44-46]. These resistance mutations may also revert to wild-type virus with time, but still some resistant viruses revert very slowly[47]. In addition, resistant variants exist, at different levels, before treatment. First, the NS3/4 protease is polymorphic in sites associated with resistance between HCV genotypes 1-6, and between some subtypes. For instance, variations in NS3-V170 are present in most HCV genotype 1 isolates, and polymorphism in NS3-D168 is characteristic of HCV genotype 3[32]. As the development of NS3/4A PIs was based in HCV genotype 1, subtype 1b, their antiviral activity with non-1b genotypes, may be not as effective, although some PIs inhibit more than one HCV genotype[22,42,48-50]. Currently neither Boceprevir nor Telaprevir should be used in patients infected with HCV genotypes other than 1. First-generation PIs have some activity in HCV genotypes 2 and 4, but very limited activity in genotype 3-infected patients[51]. Among PIs in clinical development, Simeprevir showed potent activity against HCV genotype 1, but lesser activity against genotypes 2, 4, 5, and 6[50].

Second, even within HCV genotype 1, large studies in several countries have found resistance in STAT-C naive patients (prevalence up to 5.5%)[33-35]. Thus, naturally-occurring polymorphisms can modify the treatment

response to STAT-C. In addition, there are differences in the genetic barrier to resistance between viral subtypes. HCV subtype 1a has a low genetic barrier for approved PIs, and this is the reason for higher viral breakthrough rates and selection of resistant variants observed in patients infected with subtype 1a during treatment with Boceprevir and Telaprevir. The resistance mutation R155K emerges from a single nucleotide substitution in subtype 1a viruses; whereas two different substitutions are needed in the subtype 1b viruses[17,18]. Viral breakthrough and relapse after treatment with Simeprevir is usually associated with signature resistance mutations at NS3 positions 80, 122, 155, and/or 168 (positions 80 and 168 are polymorphic between subtypes), but the distribution of mutations also varies significantly between subtypes 1a and 1b[52]. For Asunaprevir, the primary NS3 protease substitutions associated with high-level resistance identified *in vitro* occur predominately at the polymorphic amino acid residue D168 (D168A/G/H/V/Y). In addition, in single- and 3-d multiple-ascending-dose studies in HCV genotype 1a- or 1b-infected patients, a predominant pre-existing NS3 baseline polymorphism (NS3-Q80K) had ambiguous effects, but no clinically-relevant resistance-associated variants emerged in these clinical studies[53]. Finally, the large turnover and population size, together with the high mutation rate of the virus implicates that HCV variants resistant to new DAAs may be present as minority species (not detectable by direct population sequencing) within the complex pool of viral genomes circulating in a single patient[33,54,55]. Pre-existing variants resistant to Boceprevir or Telaprevir may impair virologic response before treatment is initiated[56,57]. The role of naturally-occurring polymorphisms and minority variants in treatment failure is just being elucidated, and clinical development of STAT-C compounds with pan-genotypic activity is needed.

HCV resistance to NS5A inhibitors

The HCV NS5A genomic region encodes a serine phosphoprotein of 448 amino acids (a.a. 2421-3011 of the polyprotein, numbering on HCV-H77-1a strain), which seems to have a role in transcriptional activation and participates in enhancing viral replication. NS5A has been linked to interferon sensitivity, and includes a variable region (V3), and PKR and zinc binding domains[26]. NS5A replication complex inhibitors undergoing clinical trials include Daclatasvir (BMS-790052, Bristol-Myers Squibb) and ACH-2928 (Achillion)[12]. Daclastavir is a potent oral NS5A inhibitor, studied in combination with the NS3 PI Asunaprevir alone (n = 11), or plus Peg-IFNα + RBV (n = 10) for 24 wk in genotype-1 infected patients[58]. Double and quad combination therapy produced SVR in 36% and 90% of patients respectively. In the double therapy group, viral relapse occurred in one patient (HCV subtype 1a). An analysis of baseline samples revealed a preexisting NS3 variant (R155K) conferring resistance to Asunaprevir at the time of viral relapse, whereas the NS5A resistance variant Q30E was detected only at relapse. All patients with viral breakthrough (n = 6, 55%) were infected with HCV subtype 1a. There was no resistance variants at baseline and resistance variants to both Daclatasvir and Asunaprevir had emerged in all cases by the time of viral breakthrough. Viral variants in the NS5A domain included Q30R, L31 M/V, and Y93C/N; and variants in the NS3 protease included R155K and D168A/E/T/V/Y[58]. Another phase Ⅱa study in Japan examined the combination of Daclastavir with Asunaprevir, without Peg-IFN and RBV (n = 10, HCV genotype 1b). All the nine patients who completed the full course of treatment, achieved SVR (HCV-RNA was negative at weeks 12 and 24), and there as no viral breakthrough[59]. In a survey in Japan, resistance mutations to Daclatasvir NS5A-L31M and/or Y93H were detected in 11.2% of 307 untreated patients with HCV subtype 1b infection, and Y93H (8.2%) was more prevalent than L31M (2.7%)[60]. Fifteen patients (4.9%) were infected with NS3-protease variants harboring V36A, T54S, Q80R or D168E, resistance mutations. While mutations conferring resistance to Daclatasvir or to NS3 inhibitors were frequent in this treatment-naive study population, double mutants with possible resistance to both drugs were rare. In addition, the cross-genotypic activity of Daclatasvir is under investigation. Thus, there is a rationale for expanding these double or quad therapy regimes including Dacaltasvir, but potential differences in efficacy between subtype 1a and 1b viruses should be further explored.

HCV resistance to NS5B polymerase inhibitors

The HCV NS5B genomic region encodes a 66 kDa protein composed of 591 amino acids (a.a. 2421-3011 of the polyprotein, numbering on HCV-H77-1a strain): an RdRp. The HCV RdRp resembles other viral polymerases, with a GDD motif and a right hand structure with palm, fingers and thumb domains[61]. The polymerase replicates the viral genome, in the catalytic sites located at the palm domain, from a negative strand RNA template intermediate, using an active triphosphate (NTP) as primer[62]. Depending on their chemical structure and mechanism of action, specific NS5B inhibitors can be divided into two groups: nucleoside/nucleotide analogues (NA) and non-nucleoside inhibitors (NNI). NA are alternative NTP substrates for the polymerase, forming a structure in the catalytic site that prevents the addition of new NTPs, resulting in premature chain-termination of nascent RNA[61]. The NNI inhibitors, rather than competing with NTPs, act by blocking the enzyme in the initiation of replication, preventing the conformational change necessary to elongate the nascent new copy of the RNA viral genome[63]. Four different allosteric NNI binding sites have been identified: NNI-Site A/thumb 1, NNI-Site B/thumb 2, NNI-Site C/palm 1, and NNI-Site D/palm 2; which are targets for benzimidazoles, dihydropyrones and thiophenes, benzothiazidines, and benzofurans, respectively[61]. Although the *NS5B* gene is less variable than other parts of the HCV genome, viral genetic polymorphism and mutation may also limit the

efficacy of NS5B-specific inhibitors[19,35,64,65].

The nucleos(t)ide inhibitors usually act at the catalytic site of the RdRp, where the GDD motif is located. In the replicon model, 2'-C-methyl-nucleosides select for the NS5B-S282T change, and some nucleotide inhibitors select for several other changes (S15G, R222Q, C223Y, C223H, L320I, V321I)[61,66,67]. However, a combination of at least three changes (S15G/C223H/V321I) was required to confer a high level of resistance[68]. NA inhibitors show, in fact, a high genetic barrier for the emergence of resistance[69], and no resistance mutations have been observed in NA monotherapy[70], or in combination with a PI[71]. In addition, in combination with Peg-IFNα + RBV, no viral breakthroughs due to resistance mutations could be identified[70]. NA inhibitors are therefore the most promising drugs due to the limited number of resistance mutations described[11], and the low frequency of resistant viruses circulating in the population[19,72]. Unfortunately, drugs in development in this class are few, compared to NNI.

NNI exhibit a different range of resistance profiles depending on the target site in the NS5B RdRp. Numerous substitutions associated with resistance to NNI were found mainly in the four allosteric sites A, B, C and D. Some particular changes seem extremely important for resistance, such as NS5B-M423V/I, which increases 31 times the resistance to the drug AG-02154[65]. In addition, secondary mutations may increase resistance profiles. When combined with the substitutions T19P, M71V, M423V or A442T, the change NS5B-A338V causes an increase of up to 17 times the resistance to dihydropyrones and Thiophene[73]. Variable responses to NNI may be due to natural variation in baseline susceptibility[74]. The NS5B-C316N change is frequent in HCV subtype 1b[32], and together with NS5B-Y448H, NS5B-D559G or NS5B-Y555C can increase resistance to benzofurans 30 fold.

NS5B polymerase inhibitors undergoing clinical trials include Mericitabine (RG-7128, Roche), PSI-7977 and Tegobuvir (Gilead), INX-189 (Inhibitex), Filibuvir (PF-00868554, Pfizer), VCH-222 (Vertex), ABT-072 (Abbott), and Setrobuvir (ANA-598, Anadys)[12]. Among these, nucleos(t)ide analogs such as RG-7128, PSI-7977 and INX-189 display a high genetic barrier to resistance and can be efficacious against several HCV genotypes, while non-nucleosidic inhibitors (Filibuvir, VCH-222, ABT-072, Setrobuvir, Tegobuvir) normally display a lower genetic barrier and a genotype-dependent antiviral effect (Table 1)[70,75].

CONCLUSION

Specifically-targeted antiviral therapies for HCV are entering the clinics, and more than 50 compounds are in development. The first major targets are NS3/A protease and NS5B polymerase, but other viral and host targets are in of drug development, such as the viral NS3 helicase, NS4A and NS5A proteins, or cyclophyllin inhibitors and modulators of the innate immune response. In the near future, the rate of sustained virological response will be much greater than with Peg-IFNα + RBV treatment. Due to the selective pressure of DAAs, resistant viruses circulating in the infected population can lead to treatment failure. A single mutation in the coding sequence of the viral enzyme may be sufficient to confer different levels of resistance to a particular drug. The emergence of these resistant variants has been generally observed soon after starting treatment, especially in monotherapy, as a result of the rapid decline of wild-type virus and the dominance of preexisting minority variants. Therefore, the selection of resistant viral strains can compromise new STAT-C regimens, and clinicians must take into account this problem.

In clinical trials with a single STAT-C drug, viral isolates from patients with treatment failure have exhibited one, or more than one, resistance mutation and some of them lasted for years. These results indicate that the success of new treatments using a single STAT-C may be compromised, and will require a high genetic barrier to resistance, optimal drug exposure and strict adherence. From a Public Health perspective, the allocation of resources needs to be maximized to treatment regimens ensuring success; this will depend on the effectiveness of drugs inhibiting all viral variants, minimizing the emergence of mutations. Maximum effectiveness to prevent the emergence of resistance will probably rely on a combination of DAAs with a high genetic barrier to inhibit different viral targets simultaneously, ideally with each inhibitor linked to a different set of resistance mutations.

Several combinations of DAAs, with or without concomitant Peg-IFNα + RBV administration, have obtained different efficacies in controlling HCV replication, with those regimens including compounds with a higher barrier to resistance [like nucleos(t)ide analogues, or NS5A inhibitors] being the more promising in minimizing the emergence of resistance[58,71,76]. To ensure the success of future treatments, it will be important to evaluate the true frequencies of naturally-occurring substitutions that may confer resistance to new DAA's in HCV isolates circulating in the infected population. At the Public Health level, it may be interesting to reinforce epidemiological surveillance to obtain specific data on the geographical distribution of HCV genotypes/subtypes and their prevalence in different cohorts of infected patients, because of the differential response to currently approved and new STAT-C treatment regimes in development. Strategies for increasing (or limiting) access to new treatments may require different approaches in different geographical regions. In addition, active HCV resistance surveillance is needed. Genotype sequencing on viral breakthrough (and also prior to treatment initiation) of the HCV genomic regions targeted by DAAs may be useful to identify resistance pathways, particularly in those patients in whom Peg-IFNα + RBV therapy has failed; the first candidates for newer STAT-C regimens.

Finally, the future availability of highly potent STAT-C combinations can potentially decrease the global burden

of HCV disease, and pave the way for HCV eradication. However, the high cost of STAT-C drugs, their limited efficacy in non-1 HCV genotypes, the emergence of resistance, and the need for sophisticated monitoring of new treatments makes them unreliable for resource-limited countries where the highest prevalence of chronic infection is concentrated. Clinical development of simple and affordable all-oral combination therapeutic regimes with antiviral activity to all HCV genotypes is required, and the development of an effective vaccine is still a relevant unmet goal.

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Acknowledgments

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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EDITORIAL

Reprogramming the host: Modification of cell functions upon viral infection

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Abstract

Viruses and their hosts have co-evolved for million years. In order to successfully replicate their genome, viruses need to usurp the biosynthetic machinery of the host cell. Depending on the complexity and the nature of the genome, replication might involve or not a relatively large subset of viral products, in addition to a number of host cell factors, and take place in several subcellular compartments, including the nucleus, the cytoplasm, as well as virus-induced, rearranged membranes. Therefore viruses need to ensure the correct subcellular localization of their effectors and to be capable of disguising from the cellular defensive mechanisms. In addition, viruses are capable of exploiting host cell activities, by modulating their post-translational modification apparatus, resulting in profound modifications in the function of cellular and viral products. Not surprisingly infection of host cells by these parasites can lead to alterations of cellular differentiation and growing properties, with important pathogenic consequences. In the present hot topic highlight entitled "Reprogramming the host: modification of cell functions upon viral infection", a number of leading virologists and cell biologist thoroughly describe recent advances in our understanding of how viruses modulate cellular functions to achieve successful replication and propagation at the expenses of human cells.

Key words: Virus-host interaction; Pathogenesis; Post translational modification; Viral factories; Cancer; Differentiation; Human immunodeficiency virus; Hepatitis C virus; RNAi

Core tip: Viruses are obliged intracellular parasites causing million causalities every year. In order to successfully replicate their genome, viruses need to usurp the biosynthetic machinery of the host cell. Depending on the complexity and the nature of the genome, replication might involve or not a relatively large subset of viral products, in addition to a number of host cell factors, and take place in several subcellular compartments, including the nucleus, the cytoplasm, as well as virus-induced, rearranged membranes. We describe recent advances in our understanding of how viruses modulate cellular functions to successfully replicate at the expenses of human cells.

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Viruses and their hosts have co-evolved for million years. In order to successfully replicate their genome, viruses need to usurp the biosynthetic machinery of the host cell. Depending on the complexity and the nature of the genome, replication might involve or not a relatively large subset of viral products, in addition to a number of host cell factors, and take place in several subcellular compartments, including the nucleus, the cytoplasm, as well as virus-induced, rearranged membranes. Therefore viruses need to ensure the correct subcellular localization of their effectors and to be capable of disguising from the cellular defensive mechanisms. In this hot topic highlight entitled "Reprogramming the host: Modification of cell functions upon viral infection", we describe recent advances in our

understanding of how viruses modulate cellular functions to achieve successful replication and propagation at the expenses of human cells.

The first review of this issue, by Amberkar *et al*[1] "High-throughput RNA interference screens integrative analysis: Towards a comprehensive understanding of the virus-host interplay", based on bioinformatic and statistical approaches, explains how high throughput technologies can help unveiling the complex relationship between viruses and host cell proteins, which might represent targets for potential therapeutic intervention.

In "Architecture and biogenesis of plus-strand RNA virus replication factories", Paul *et al*[2] propose an innovative classification of positive strand RNA viruses according to the morphology of membrane rearrangements they are able to induce, and on which genome replication is believed to take place. The interplay of viral and cellular factors in the biogenesis of these replication factories is discussed.

The relationship between viruses and the host cell defensive system is the particular focus of the two following reviews "Innate host responses to West Nile virus: Implications for central nervous system immunopathology", by Rossini *et al*[3] and "Paramyxovirus evasion of innate immunity: Diverse strategies for common targets" by Audsley *et al*[4], which both provide a simultaneously accurate and concise summary of viral strategies to subvert the innate response at the molecular level, and the implication thereof in viral mediated pathogenesis.

In the following review "Viral proteins and Src family kinases: Mechanisms of pathogenicity from a 'liaison dangereuse'", Pagano *et al*[5], describe the mechanisms by which several viruses exploit protein-protein interactions to modulate the subcellular localization and enzymatic activity of these cellular enzymes, thus promoting their replication and regulating cell survival. Indeed, viruses are known to efficiently modulate the cell post translational machinery for their own benefit. Similarly, Mattoscio *et al*[6], in "Viral manipulation of cellular protein conjugation pathways: The SUMO lesson", review the relationship between the Small Ubiquitin like MOdifier apparatus and a number of DNA and RNA viruses.

"Effects of human immunodeficiency virus on the erythrocyte and megakaryocyte lineages" by Gibellini *et al*[7], deals with the ability of human immunodeficiency virus-1 infection to affect the differentiation potential of different cellular types, including osteoclast and vessel stem cells, and its implication in pathogenesis.

Finally, the hot topic highlight is closed by an intriguing hypothesis formulated by Avanzi *et al*[8], "How virus persistence can initiate the tumorigenesis process" describing in detail how infection-reinfection/reactivation cycles of viruses might contribute to the initiation of tumorigenesis.

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TOPIC HIGHLIGHT

Gualtiero Alvisi, PhD, Assistant Professor, *Series Editor*

High-throughput RNA interference screens integrative analysis: Towards a comprehensive understanding of the virus-host interplay

Sandeep Amberkar, Narsis A Kiani, Ralf Bartenschlager, Gualtiero Alvisi, Lars Kaderali

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Abstract

Viruses are extremely heterogeneous entities; the size and the nature of their genetic information, as well as the strategies employed to amplify and propagate their genomes, are highly variable. However, as obligatory intracellular parasites, replication of all viruses relies on the host cell. Having co-evolved with their host for several million years, viruses have developed very sophisticated strategies to hijack cellular factors that promote virus uptake, replication, and spread. Identification of host cell factors (HCFs) required for these processes is a major challenge for researchers, but it enables the identification of new, highly selective targets for anti viral therapeutics. To this end, the establishment of platforms enabling genome-wide high-throughput RNA interference (HT-RNAi) screens has led to the identification of several key factors involved in the viral life cycle. A number of genome-wide HT-RNAi screens have been performed for major human pathogens. These studies enable first inter-viral comparisons related to HCF requirements. Although several cellular functions appear to be uniformly required for the life cycle of most viruses tested (such as the proteasome and the Golgi-mediated secretory pathways), some factors, like the lipid kinase Phosphatidylinositol 4-kinase Ⅲα in the case of hepatitis C virus, are selectively required for individual viruses. However, despite the amount of data available, we are still far away from a comprehensive understanding of the interplay between viruses and host factors. Major limitations towards this goal are the low sensitivity and specificity of such screens, resulting in limited overlap between different screens performed with the same virus. This review focuses on how statistical and bioinformatic analysis methods applied to HT-RNAi screens can help overcoming these issues thus increasing the reliability and impact of such studies.

Key words: RNA interference; High-throughput; Cell population; Dependency factors; Bioinformatics; Human immunodeficiency virus; Hepatitis C virus; Dengue virus; Viral infection; Virus-host interactions

Amberkar S, Kiani NA, Bartenschlager R, Alvisi G, Kaderali L. High-throughput RNA interference screens integrative analysis: Towards a comprehensive understanding of the virus-host interplay. *World J Virol* 2013; 2(2): 18-31 Available from: URL: http://www.wjgnet.com/2220-3249/full/v2/i2/18.htm DOI: http://dx.doi.org/10.5501/wjv.v2.i2.18

INTRODUCTION

Viruses are obligate intracellular parasites causing more

than 3 million deaths per year worldwide (http://www.cdc.gov/). Development of highly efficient vaccines to prevent infection, or antiviral compounds to promote viral clearance from infected patients is hindered by their high variability and mutation rate[1]. Given the small size of their genome, which can be as small as just a few kilobases[2], viruses necessarily rely on host cell factors (HCFs) in order to propagate their genetic information. Therefore key HCFs required for the viral life cycle might represent potential target for the development of new anti-viral compounds[3]. Indeed these factors can be ablated either pharmacologically or genetically, resulting in a drop of viral replication[4,5]. While pharmacological ablation necessarily relies on the availability of highly specific inhibitors, the discovery of RNA interference (RNAi) allows to genetically hinder the expression of virtually any human gene, by reducing its mRNA levels and therefore protein expression[6,7]. The availability of libraries of small interfering RNAs (siRNAs) directed towards almost every human gene (genome-wide libraries) enables to perform large scale, high-throughput RNAi (HT-RNAi) screens to identify key HCF involved in virtually any measurable cellular process. To this end, HT-RNAi technology has been extensively used to identify cellular factors involved in cell division[8], Wnt signaling[9], Janus kinase/signal transducers and activators of transcription signaling[10], extracellular signal-regulated kinases signaling[11], caspase activation[12], mitochondrial function[13] and many others. A similar approach could also be undertaken to search for HCFs required for a certain step of the life cycle of any given virus, which is able to replicate in cell culture. Because viral infection is a multi-step process that starts with the interaction between the parasite and the target cell and ends with the release of newly generated infectious particles, any of these steps is a potential target of therapeutic intervention through silencing of the involved HCFs. Therefore, several genome-wide HT RNAi screens have been performed to identify key factors involved in the life cycle of a number of viruses, including human major pathogens such as influenza virus (INF)[14-16], human immunodeficiency virus-1 (HIV-1)[17-19], and human hepatitis C virus (HCV)[20,21], the only constrain being the availability of a robust cell culture system to assay the outcome of infection. The very first genome-wide HT-RNAi screen performed on viruses was performed on Drosophila C virus (DCV)[22]. Indeed most of the first genome wide HT-RNAi screens were performed in Drosophila cells because of several reasons, including the fact that Drosophila Melanogaster's genome was completely sequenced in 2000[23], allowing for synthesis of comprehensive Drosophila dsRNA libraries[24,25] and that long dsRNAs added to the medium of Drosophila tissue culture cells are rapidly taken up by the cells in the absence of any transfection reagents, mediating efficient and specific mRNAs knockdown[26]. The first genome-wide screen for viral HCFs relied on a very simple experimental set-up: cells were incubated with a single RNAi specific for each gene in 384 well plates for 3 d, infected with DCV, and 1 d later, processed for immunofluorescence against the capsid antigen before automated microscopic imaging. By visual inspection, the authors identified 210 dsRNA species that reduced the relative number of infected cells by > 40%. dsRNAs targeting these genes were re-synthesized and tested again for their ability to decrease DCV infection. This "validation" screening allowed identifying 112 host dependency factors (HDFs). Among them, 66 proteins were ribosomal proteins, specifically required for translation of DCV polyprotein but not for vesicular stomatitis virus, a pathogen whose genome, in contrast to that of DCV, does not contain a ribosomal entry site (IRES) mediating RNA translation in the absence of a 5' cap. The authors therefore concluded that the ribosomal genes identified in their study are essential for DVC IRES mediated genome translation.

Since this very first example, it became rapidly clear that many sources of errors such as RNAi reagent design, an inhomogeneous staining, differences in cell growing properties as well as in transfection and infection efficiencies could negatively affect the outcome of such HT-RNAi screens. Therefore, HT-RNAi screens became more and more sophisticated (Table 1). Authors started to worry to strengthen the statistical reliability of their studies by including several replicas, and increasing the number of oligos tested per gene. The most popular approach so far has been to test four different oligos per gene, pooled in a single well in a primary screen, to reduce the so called "off targets effects"[27]. Subsequently hit genes from the primary screen are further tested in a secondary validation screen, where the four different oligos used in the primary screen are tested individually for their ability to reproduce the original phenotype[17,20,21,28]. Several studies started to include the possibility to distinguish genes important early in the viral life cycle (entry/replication phases) from those involved later on (assembly/release of new viral particles), by implementing a two-step procedure, according to which cells are incubated with the siRNA library before infection with the virus of interest. Measurement of viral replication at a given time point enables to identify gene products important for early phases of the viral life cycle such as virus entry and genome replication. Simultaneously, supernatants are collected from infected cells and used to re-infect naive cells, therefore enabling to identify genes important for late stages of viral life cycle such as viral assembly and release[14,17,20]. As far as the readout is concerned, some authors preferred to utilize reporter viruses carrying either the GFP[29]-to avoid issues related to antigen staining and detection - or the Luciferase (Luc) genes in their genome[15,16,18,21], the latter solution enabling an easier and more quantitative analysis of the levels of viral replication. Interpretation of HT-RNAi screening results is also complicated by the fact that different screens performed with the same virus yielded little overlap between HCFs, raising questions concerning the reliability and reproducibility of this approach[30]. Hence, several authors have implemented interesting bioinformatics and statistical approaches (see below) to strengthen the significance and

reliability of their results by integrating RNAi data with protein-protein interaction (PPI) databases[18,20].

Overall the picture emerging from the above-mentioned studies is that different viruses rely on some common structures, such as the proteasome proteolytic pathway, the spliceosome complex, and the Golgi secretory system. Because of the "housekeeping" nature of the latter processes, these findings, although representing a crucial stating point to understand the molecular biology behind the virus-host cell interaction, might be of limited importance to the identification of anti-viral targets and to the understanding of how specific viruses differentially exploit the cell for their own purposes. However, a few virus specific HCFs have also been identified. Among them, a lipid kinase, the phosphatidylinositol-4-kinase Ⅲα (PI4K-Ⅲα) has been identified by several HT-RNAi screens as a crucial factor for HCV replication, in spite of differences in the HCV genotypes used and the experimental setup[20,21,31-33]. A recently proposed model hypothesized that during HCV infection, a viral protein recruits PI4K-Ⅲα to the sites of viral replication to increase local levels of phosphatidylinositol-4-phosphate, necessary for their integrity of the membraneous replication compartment and hence viral replication[34]. Importantly, a recent study reported that AL-9, a 4-anilino quinazoline specifically inhibiting HCV replication[35], acts a selective inhibitor of PI4K-Ⅲα[36]. This inhibitor could therefore represent the basis for the development of new-highly needed antiviral compounds to combat HCV infection. The next sections offer a brief overview on how bioinformatics and statistical approaches can overcome most limitations connected with HT-RNAi screens applied to the study the virus-host interaction, resulting in a simple workflow for the analysis of HT-RNAi screens aimed at identifying key host regulators of viral life cycles (Figure 1).

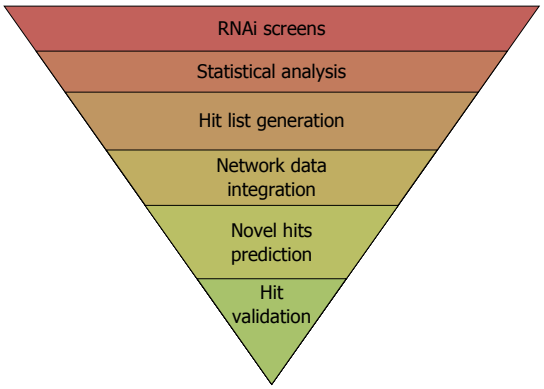

Figure 1 A schematic workflow for the analysis of high-throughput RNA interference screens to identify host cell regulators of viral life cycle.

FROM EXPERIMENTAL SET-UP TO "HIT CALLING": STATISTICAL ANALYSIS OF HT-RNAi SCREENS

Readout systems for HT RNAi screens are extremely heterogeneous, ranging from bulk readouts of fluorescence reporters to high-content microscopy based assays. Basically any phenotype, either directly or indirectly measured through a reporter, can be used as readout in HT-RNAi screens. However, the main measurement types of cell-based screens in use are: Uniform well readouts: these assays usually use high throughput plate readers to produce their measurements. Absorbance, Luminescence, Fluorescence Intensity, Fluorescent Polarization and Resonance Energy Transfer are the most usual uniform well detection methods[37]; Reporter gene systems: these are mostly high throughput assays using Fluorescence-assisted cell sorting. They employ high throughput FACS to produce readouts of GFP, Luc, *etc.*[38]; High-Content Imaging Screens: they are designed to identify those genes that alter the cellular phenotype in a desired manner (*i.e.*, decreases in the production of cellular products, nuclear and cellular morphology, proteins subcellular localization, *etc.*)[37,39].

The selection of the type of assay depends on the goal of screen and practical constrains. The analysis of arrayed screens can involve application of image analysis software or custom programs, as well as various methods of statistical analysis and Bioinformatics (see below). The aim of statistical analysis is to identify "hit" genes that are robust up- and down-regulators of viral replication. Overall, most of the methods currently used for statistical analysis of RNAi screens are reminiscent of those developed in the past for the statistical analysis of cell-based small-molecule screens, with considerable improvement having been implemented in several aspects - including data normalization, replicate tests, and selection of cut-off threshold to determine hits[40]. Cell death and cell clumping are among the most serious problems, which can be directly or indirectly linked to the silencing effect of individual siRNAs[39]. These phenomena can create background or saturation effects in the corresponding wells. In addition, viral infection might induce even more data variation since it can lead to a different cellular behaviour[41]. Errors of unknown origin may also occur over the entire process. These adverse effects can often be minimized by quality control procedures and statistical corrections. Data variation caused by stochastic reasons can be minimized performing additional experimental replicas.

Successful data analysis heavily depends on careful experimental design and assay development prior to the primary screen[42]. Therefore, for example, the optimization of transfection conditions is crucial to the success of experiments. Several factors play important roles in the development of a good assay. The nature of RNAi duplexes to be used (different companies offer RNAi with specific chemical modifications reducing off-target effects), the number of unique individual RNAi duplexes targeting each gene, the number of replicate tests, the number of "no treatment" controls (negative controls), the individual plates layout design (including the placement of negative controls) are the most important factors which should be considered during the experimental design[38,43,44]. Testing of two or more non-overlapping

Table 1 List of high-throughput RNA interference screens performed to identify host factors involved in viral life cycle

Family	Virus	Readout	Viral life cycle step(s) studied	Partition (primary/validation)
Flaviviridae (ssRNA+)	DENV[1][100]	IF	Single step	Primary: single oligo; validation, single oligo (the same)
Picornavirus (ssRNA+)	DCV[1][22]	IF	Single step	Primary: single, validation, single (the same)
Flaviviridae (ssRNA+)	HCV[21]	Luc (reporter virus)	Single step (replicon)	Primary: pools of 4 oligos; validation: the 4 oligos forming the pool tested individually
Flaviviridae (ssRNA+)	HCV[20]	IF	Two step (full virus): entry/replication; assembly/release	Primary: pools of 4 oligos; validation: the 4 oligos forming the pool tested individually
Retroviridae (dsRNA)	HIV-1[17]	Part Ⅰ: IF; part Ⅱ: Luc (reporter cell line)	Two steps: entry/replication; assembly/release	Primary: pools of 4 oligos; validation: the 4 oligos forming the pool tested individually
Retroviridae (dsRNA)	HIV-1[18]	Luc (reporter virus)	Single step	Primary only: 3 pools of 2
Retroviridae (dsRNA)	HIV-1[19]	Beta-Gal activity	Two steps, but without distinction between them	Primary: pools of 3; validation; independent poools of 3
Orthomixoviridae; (ssRNA- segmented)	INF[14]	Part Ⅰ: IF; part Ⅱ: Luc (reporter cell line)	Two steps: entry/replication; assembly/release	Primary: 4 oligoes
Orthomixoviridae; (ssRNA- segmented)	INF[15]	Luc (reporter virus)	Single step	Primary only: some genes 2 oligoes, other genes only one
Orthomixoviridae; (ssRNA- segmented)	INF[1][16]	Luc (reporter virus)	Single step	Primary only: a single sirna x gene
Rhabdoviridae (ssRNA-)	VSV[29]	IF	Single step	Primary: 2 pools of 2 oligos; validation: pool of 4 oligos from different vendor
Flaviviridae (ssRNA+)	WNV[28]	IF	Single step	Primary: pools of 4 oligos; validation: the 4 oligos forming the pool tested individually

[1]Drosophila cells. HIV: Human immunodeficiency virus; IF: Immunofluorescence; Luc: Luciferase; DENV: Dengue virus; DCV: Drosophila C virus; HCV: Hepatitis C virus; VSV: Vesicular stomatitis virus; WNV: West Nile virus.

RNAi reagents per gene is nowadays a general standard for primary screens[38]. Once RNAi concentration and transfection conditions have been established, checking the sensitivity to accurately differentiate between positive "hits" and negative controls can be very useful[45]. During this hit selection process, two kinds of decision errors can occur, leading to "false positives" (FPs), experimental findings that cannot be subsequently confirmed, and to "false negatives" (FNs), factors which should have been identified were not. If the assay is not sensitive enough, a high frequency of FNs will be obtained. Conversely, if the readout is too sensitive, a significant number of FPs will be identified. The best way to ascertain the rates of FNs and FPs is to perform a pilot screen. For this purpose, two or more plates fully loaded with positive and negative controls should be used to test the outputs "robustness"[46]. Three measurements are commonly used to this end: signal-to-background ratio, coefficient of variation (CV) and the Z' factor[45]. As assay variability increases, the signal-to-background ratio must increase for a screen to be successful.

Some candidates identified through a screen might generate the phenotype of interest; however, this might be due to the type of assay used for the readout or to an off-target effect. To overcome such problems, one can use an alternative, or orthogonal, screening procedure. The selected candidate forms the basis for further investigations, for example a secondary screen (also called validation screen). Secondary screens test a much smaller number of compounds (*e.g.*, the 1% strongest hits from the primary screen) and typically use at least duplicate measurements. The magnitude of the statistical artefact can be minimized, *e.g.*, by obtaining replicate measurements, and thus improving precision of the overall estimate. The assumptions that RNAi duplexes targeting specific genes randomly plated and the most of them do not have an effect on viral replication for secondary screens are not valid. Below, we present a sample workflow for analyzing RNAi screens.

Quality control

The goal of HT-RNAi screens is normally to identify "hits". To this aim, it is of crucial importance to separate FPs from bona fide "hits". This is largely related to the quality of the assay used. It is therefore necessary to monitor each step, checking the quality of raw and normalized data. To increase the probability of success, quality assessment should be performed while the screen is in progress, and also after each step of the analysis pipeline, thus allowing the detection of potential issues as they occur. This will also help with the choice of analysis methods. In case of a failed quality control for individual wells or plates, these should be either removed from further analysis or repeated.

In biological experiments the use of controls - positive and negative - helps to assess the quality of obtained data. Negative controls can be used to assess plate-to-plate variability, and provide a means to measure background noise levels of an assay. Positive controls provide an estimate of expected effect strengths, and are used to establish if effects are observed at all, and if they are of the expected strength. Controls allow the calculation of several different quality metric such as signal to noise ratios, the dynamic range[47], CV or the Z' factor. In contrast to simple signal/noise ratio, the dynamic range and the Z' factor calculate the separability of positive and negative

controls and use this criterion to evaluate assay quality. CV measures the data quality based on the reproducibility of results. In contrast to dynamic range and the Z' factor, CV does not use controls and can be used in case that controls are not available or they did not work properly in some screens or at least on individual plates. Calculating the correlation among replicates by correlation measures such as Pearson's correlation or Spearman's rank also can be used to check the reproducibility and reliability of the data.

Apart from quality metric, plate visualization is one of the most effective techniques to find systematic sources of error or identify data with poor reproducibility due to suboptimal assay design or implementation[37,39,42,43,48,49]. Heat maps and plate-well scatter plots, which allow to display the overall screen performance, as well as replicate correlation plots to visualize overall reproducibility, are the most widely used methods used for plate visualization[43,48]. Box Plots of readouts of all plates can be used to detect systematic errors among the plates.

When RNAi duplexes are randomized between plates and experiments are performed under identical conditions, the box plots of raw data should show approximately the same location and scale. However, it is possible due to systematic variability that some of the plates have lower (higher) median intensities than the others, resulting in considerably higher (lower) hit rates on these plates. This can be the consequence of pipetting issues resulting in altered transfection or infection efficiencies: such deviations can be adjusted by normalization. For more details about the individual plots and their interpretation, please refer to[47,48,50,51]. Finally, wells with lowest and highest 1%-5% of cell counts in the entire screen are sometimes excluded from further analysis in particular in the case of image based screens, because of potential interference with viral replication readout and errors in image segmentation when cells are very dens[39].

Data normalization

Readout of each spot in a plate is a function of at least two factors: the siRNA's real activity and random error. There are many sources of systematic errors (variations) that affect readouts of HT-RNAi screens. The ability of combination and comparison of all of the plates in a production run to each other is very important. Systematic errors can cause a high degree of intra-plate and plate-to-plate variability, which does not allow comparison and combination of data from different plates. Data normalization is a process intended to remove such variation from the data to allow comparison and combination of data from different plates of the screen. Intra-plate spatial effects and correlation between cell numbers and signal intensity are the most important sources of systematic errors[40]. A number of normalization methods have been developed to address these issues[42,47]. Normalization is generally performed at two levels: per-plate and per-experiment (intra- and between-plates normalization).

The per-plate (intra-plate) normalization aims at reducing systematic errors on individual plates, such as differing cell numbers over the plate, or edge-effects affecting the signal intensities. This can be the consequence of pipetting issues resulting in altered transfection or infection efficiencies, as well as of evaporation of media from the outer wells. The per-experiment (between-plate) normalization removes systematic bias that occurs between different plates. This bias might be due to measurements performed using diverse microscope settings or under different environmental situations such as different levels of humidity. Since data is varying across specific experimental setups, a standard normalization strategy that is appropriate for all of them does not exist. For example, normalization methods for primary and validation screens are different, due to the method of selection and distribution of the RNAi duplexes. Normalization methods can be categorized into two main groups: control-based and sample-based. Control-based normalization methods compare individual experimental sample values to aggregated values of negative controls, while sample-based method use the samples themselves as *de facto* negative controls[42]. The latter choice can provide more accurate measurements, because on each plate the number of experimental samples exceeds that of the negative controls by several folds. This approach is based on the assumption that most experimental samples will not display a biological effect in the assay being analyzed. Obviously, this assumption is not valid in the case of validation screens and therefore sample-based normalization methods should not be used in the case of validation screens. In this case, plates are made comparable by control-based normalization method. Additionally, the use of sample-based normalization methods is particularly problematic when dealing with statistical measures (such as mean and standard deviation) that are strongly sensitive to outliers in the data.

Controls-based normalization

Including controls on every individual plate can help identifying plate-to-plate variability and establishing background levels of an assay. One common approach to for plate-to-plate normalization is to scale the intensity values based on the controls. Whether for the normalization the negative or the positive controls shall be chosen, it depends on the type of experiment. For RNAi data, negative controls are used in most cases. It should be noted that in this approach, any inaccuracies and random measurement errors in controls would lower the accuracy and precision of the normalized values through error propagation. Therefore, it is important to obtain as accurate and precise measurements as possible. Using a relatively large number of control measurements and omitting outliers among the controls before normalizing can improve the quality of normalized values.

In this approach, the mean or median of the controls of a plate is subtracted from the readout value of each spot in the same plate and the result is divided by the controls standard deviation or median absolute deviation (MAD).

Sample-based normalization

As mentioned before, under the assumption that most

siRNAs in plate would not cause an effect, it is possible to use experimental samples as controls. Z-score normalization is a well-known data scaling strategy, which uses this assumption. For each spot, the Z-score is defined as the number of standard deviations from the mean of the samples on the plate.

The readout of each spot rescaled relative to intra-plate variation by subtracting the average of the plate values and dividing the difference by the standard deviation estimated from all measurements of the plate. In this approach, the mean of all the samples on the plate is used instead of that of the negative controls, thus limiting the need for large numbers of controls. Z-score gives explicit information on the strength of each siRNA relative to the rest of the sample distribution. An advantage of Z-score is that it integrates information about the variability of replicate measurements in the score. The main disadvantage of this method is its non-robustness to outliers, that can strongly affect estimates of the mean and standard deviation used in the Z-score.

A modified version of it called the robust Z-score, is generally considered preferable for the analysis of HT-RNAi screens. It uses the median and MAD for mean and standard deviation in the Z-score calculation.

B-score normalization

The B-score is known as a robust analogue of the Z-score. It is more robust to the presence of outliers, and also differences in the measurement error distributions of the different spots on a plate. If the quality control has identified the presence of within-plate systematic errors, the B-score normalization[52] may be applied to remove row and column effects within a single plate. The systematic measurement offsets for each row and column, row and column effects, is estimated using the Tukey median polish method. The resulting residuals within each plate are then divided by their MAD to standardize for plate-to-plate variability. This thus allows the comparison of different plates, since it scales the data according to the overall plate median. The B-score has three advantages: it is nonparametric (that is, it makes minimal distributional assumptions), it minimizes measurement bias due to positional effects, and it is resistant to statistical outliers[47].

Lowess normalization

Lowess (locally weighted least squares regression) normalization performs intra-plate corrections. If RNAi data is multi-parametric, different read-outs may depend on each other and these can cause a systematic bias. Lowess regression is a technique for fitting a smoothing curve to a data. Data points that are nearer to the estimated fit are weighted higher than more distant points. The degree of smoothing is determined by the window width parameter. A larger window width results in a smoother curve, a smaller window results in more local variation. The normalized signal intensities are the difference of the signal intensity values and the corresponding point on this curve[51]. For example, Lowess normalization can be applied to remove the correlation between signal intensities and cell count by adjusting the signal intensities for the effect of unequal cell numbers in wells. This should be done for each plate individually, since effects may be different from plate to plate[39].

Population context normalization

Very recently it was shown that different cells in a population display heterogeneity in their cellular behaviours[53,54]. This heterogeneity implies that cellular responses to a particular stimulus or perturbation, such as virus uptake, may also be variable[41]. For example certain viruses prefer to infect cells that are in a less dense region, others preferentially infect densely packed cells[54]. Therefore analyzing certain phenotypes at the single-cell level instead of using population averages to measure an effect might completely change the results. Snijder *et al*[54-56] showed that the population context of a cell strongly affects its behaviour: factors such local cells density, their position within an islet, size, distance from cell-colony edges and population size are the main determinants of cell to cell variability in HT-RNAi screening. To address this issue they suggested normalizing data by considering the population context. They corrected population context effects using quantile multidimensional bin models[55]. Knapp *et al*[57] used a similar approach in normalizing data but they developed a statistical testing procedure that takes into account individual cell measurements in hit-scoring. They used gene set enrichment analysis (EA) on sets defined not by genes but by cells coming from one spot, one siRNA or one gene. These approaches suggest that normalization for population context can lead to a substantial decrease in experimental variability, and may to some extent underlie the low gene overlap and lack of reproducibility of RNAi screens targeting even the same virus.

Hit calling

Once data have been pre-processed with quality control checks and normalization procedures, the next critical step is the hit identification procedure to decide which siRNAs should be further tested in a secondary screen. The identification of "hits" or "screening positives" is the goal of any primary RNAi screen. Hit identification is, essentially, the selection process of those samples whose measured values for a given phenotype differ significantly from that of the negative controls[52]. A wide range of hit identification techniques is available. Hits can be identified as a percentage of the genes that generate the highest measured activity (*e.g.*, top 1%), or as those whose activity exceeds a fixed "percent of control" threshold. Alternatively, the hit threshold may be defined as a number of standard deviations (typically 2) beyond the mean of the raw or processed data. This approach selects a standard deviation threshold relative to the mean or median normalized data and defines "hits" the samples that go beyond this threshold. However, hits (outliers) may cause the distribution of the siRNAs measurements to be skewed. The use of the median rather than of the mean is more robust to outliers, and has been shown to more effectively enable the identi-

fication of weak hits from RNAi data[58].

The threshold methods assume a common magnitude of random error for all measurements, but do not capture data variability effectively. To address this issue, researchers then turned to the Z-score method or strictly standardized mean difference (SSMD)[59], which can capture data variability in negative controls. According to the Z-score method, any compound whose score after Z-score normalization deviates from the bulk by a given threshold will be considered as hit. The Z-score method is based on the assumption that the measured values (usually fluorescent intensity in log scale) of all investigated siRNAs in a plate have a normal distribution. SSMD also works the best under the normality assumption. The drawback common to all of these metrics is that they rely on non-robust statistics, which may lead to inferential errors in hit detection. Because of the potential existence of true hits and strong assay artefacts, outliers are not uncommon in HT screens. The regular versions of Z-score and SSMD are sensitive to outliers[59]. In general, there are two major types of approaches for hit selection: analytic metrics and hypothesis testing. The methods belonging to the first approach (such as fold change, mean difference, SSMD, percent activity, percent viability and percent inhibition) assess and rank the size of RNAi effects, while the methods belonging to the second group (for instance *t*-test) test the null hypothesis that no difference exists among the means of particular well and negative controls or mean of plate[48,52,59,60]. If enough replicates are available, a statistical approach can be applied to assign a *P* value to each condition. If the *P* value is smaller than a given significance level, the null hypothesis can be rejected. A common practice is to use the *t*-test. It is a parametric testing method (assuming normally distributed data), which assesses the difference of the means between replicates for each condition.

If siRNA duplexes are randomly distributed on a plate and if it can be assumed that most of them have no effect, replicates in the test can be compared with the overall population. If this assumption is not valid, *e.g.*, in a validation screen, the test is carried out against negative controls. This approach needs at least three replicates of each condition and that data follows a normal distribution[61]. In case of non-normal distribution, the Mann-Whitney test can be used as non-parametric test[39].

The methods for hit selection differ according to the experimental setup of the HT-RNAi screen, depending on the fact that replicates have been performed or not. For example, the Z-score method is suitable for screens where replicates have not been performed, whereas the *t*-test is suitable for screens where three or more replicates have been performed. It is not possible to directly estimate the data variability for each siRNA in screens without replication. Instead, it is indirectly possible to estimate data variability by making the assumption that every siRNA has the same variability as a negative reference in a plate in the screen. The Z-score, the B-score and the SSMD rely on this strong assumption for cases without replicates[62].

BEYOND THE SCREEN: BIOINFORMATICS INTEGRATIVE APPROACHES FOR RELIABLE HIT IDENTIFICATION

A typical outcome of any statistical analysis of a genome-wide HT-RNAi knockdown screen is a list of gene products that significantly differ from other genes in the same study, relatively to a given readout. Classically, these lists are then subjected to over-representation analysis (ORA) or EA over different known pathway datasets such as KEGG, Reactome, Wikipathways and gene ontologies (GO), in order to facilitate interpretation of the hits functional importance. A major caveat in such analyses is the fact that the datasets used for such analyses are far from being complete. Inconsistencies and lack of concurrency between these pathway databases reduces their reliability, thus hampering the coverage of ORA/EA. This problem is particularly evident in the case of HT-RNAi screens concerning the same virus[17-19], where the overlaps over these ontologies are minimal[30]. In order to overcome this problem, network approaches have been implemented to analyze HT-RNAi screens. This section describes studies that exemplify the usage of PPI network data for analyzing RNAi screens.

Integrating network data for analyzing RNAi screens

With the wealth of public repositories housing PPI data, and exponentially growing computational power to analyze such data, the need to integrate the outcome of HT-RNAi screens with PPI data is pressing. Protein interactions between viral and host proteins are a subset of this data type that can be created by combining previously published and experimentally newly identified interactions. VirHostNet[63], VirusMINT[64] and the HIV-1 Human Protein Interaction Database (HHPID) at National Institute of Allergy and Infectious Diseases (NIAID)[65] are examples of such resources.

A host of analysis pipelines has been developed to integrate PPI data with the HT-RNAi screens hits, which can be applied to add depth and significance to latter results. An example is the SinkSource algorithm described in a recent study[66]. In the latter, the authors used a semi-supervised machine learning approach to predict novel HIV-1 HDFs using known HDFs. In other words, by combining HDFs identified from recent studies[17-19] and PPI data, the authors developed a classification algorithm that would learn from the known HDFs in a network context to then predict novel ones. The host PPI network is modeled as a liquid flow network. Each node (protein) is a reservoir of fluid while an edge (connection between 2 nodes) is a pipe. The weight of an edge indicates the amount of fluid that can flow through the pipes per unit time. When the fluid network attains equilibrium (amount of liquid flowing into each node equals amount flowing out), the reservoir height at each node denotes the confidence that the node is a HDF. HDFs identified in three previously published HIV screens[17-19] were assigned a

reservoir level at of 1 unit while non-HDFs nodes had a reservoir level of 0. This algorithm is similar to the one formulated in a previous study[67] for functional prediction of genes, except that SinkSource also accepts negative values in the form of non-HDFs which are non-lethal. These non-lethal, non-HDFs formed the negative set while HDFs identified in the three studies and their intersection formed the 4 positive datasets used for prediction of novel HDFs through a two-fold cross validation. The latter involved splitting of both the positive and negative datasets in halves and each half was used for prediction of the genes in the other half. SinkSource had higher specificity and precision-recall values when compared to six similar algorithms (used for functional gene prediction). SinkSource predicted 1394 HDFs in addition to the 908 from the above three screens, with an accuracy > 80% based on two-fold cross validation described earlier. After combining the known HDFs with those predicted by their algorithm, the authors then searched for dense subgraphs in an integrated protein network through MCODE[68]. Using this approach, they identified cellular processes and components essential for HIV replication. These included, as far as the GO cellular component are concerned: spliceosome, kinetochore and mitochondrion, whereas GTPase mediated signal transduction, DNA replication initiation and MHC protein complex were identified as enriched cellular processes.

Another example of a network based analysis between PPI and HT-RNAi data is from MacPherson *et al*[69]. In this study, the authors utilized the HIV-1 HHPID at NIAID[65] and applied a bi-clustering algorithm to identify clusters of genes enriched for HIV-1-Human PPIs. In order to establish a hierarchical overview of functions from the clusters, they were further linked to form a cladogram. The distance between 2 clusters was based on the number of overlaps between them; clusters with more overlaps were closer to each other than the ones with fewer overlaps. GO enrichment of these clusters then defined the cluster function and in turn allowed identification of 37 host subsystems potentially important for HIV-1 infection. Interestingly, hits previously identified in three published HIV HT-RNAi screens[17-19] were found in 10 of the 27 subsystems identified. These included proteasome core complex, regulation of apoptosis, mRNA transport, endosome, RNA polymerase activity, peptidase activity, regulation of transcription, ubiquitin camp-dependent protein kinase complex, and v-akt. Classically, the virus-host interaction dataset used in this study would only provide information about how a viral protein interacts with a host protein and its mechanism, as extracted from literature. In this study, the authors showed how a viral protein interacts with host cellular systems in contrast to a single protein. A theoretical validation was provided by highlighting systems enriched with hits from the 3 HIV-1 RNAi screens[17-19].

A very important consequence of viral-based RNAi screens could be the discovery of new potential targets for the development of anti-viral agents. Over the years, repositories holding detailed information on various drugs, including their cellular targets, have been publicly made available. de Chassey *et al*[70] used the DrugBank database, one of such resources (http://www.drugbank.ca) to identify potential drug targets for INF. By combining results from 6 different IFV HT-RNAi screens, the authors identified 925 essential host factors (EHFs), required for IFV replication[14-16,71-73]. Network analysis performed integrating these data with the PPI dataset from VirHostNet[63] revealed that 17 EHFs are directly targeted by a viral protein while the neighborhood of EHFs (proteins physically interacting with EHFs) included 204 proteins that were targeted by at least one viral protein.

In parallel, known drug molecules interacting with EHFs were further retrieved from DrugBank. This analysis revealed that 100 EHFs could be targeted by 298 different molecules comprised of 204 FDA-approved drugs and 94 experimental drugs. These 100 EHFs were further filtered down to 33 proteins, based on their ability to fulfill at least one of the following three criteria: the EHF was directly targeted by a viral protein, the EHF was connected to at least another EHF, and the EHF was connected to a non-EHF targeted by the virus. Of these, 32 EHFs could be targeted by 49 FDA approved molecules with an exception of one target, HSP90AA1, fulfilling the first 2 criteria mentioned above, is also directly interacting with a viral protein. Interestingly, the authors found that this EHF is the target of 1 FDA-approved molecule (Ribafutin) and 5 experimental molecules. Among the latter, Geldanamycin has already been proved to reduce IFV viral replication by 2 logs in cell culture[74]. Therefore the authors concluded that combination of Geldanamycin with Ribafutin (which is also used as a first line of treatment in tuberculosis) could represent a novel strategy to identify antiviral drugs to combat IFV infection.

As with any high-throughput study, the issue of false-positives and false-negatives also exist for HT-RNAi screens. FPs due to off-target effect of RNAi are common in genome-wide screens and confer ambiguity to the final hit-list selected from a screen. Hence, it is recommended to perform a multi-level validation and a functional analysis for hit genes. Even more critical and tricky is the issue of FNsof a screen. These are typically genes that have an effect but are missed due to the statistical selection criteria. Wang *et al*[75] addressed these problems by developing an algorithm based on machine learning principles, utilizing protein interaction data and network topology. Considering network centralities such as direct neighbour, shortest path, diffusion kernel and association analysis-based transformation[76] along with gene similarities, they developed a set of scoring functions called Network RNAi Phenotype (NePhe). Utilizing the guilt-by-association principle, Wang *et al*[75], reasoned that FNs would be scored higher by a scoring function over false-positives FPs, as they would be linked by a greater number of true hits. Thus, a near-ideal gene classifier would always rank FNs with a higher rank compared to a non-hit. When this methodology was applied over the Wnt

and the Hedgehog signaling pathways, the NePhe scoring system was able to identify all regulators, which were missed even by the follow-up validation screens. This algorithm was tested on 24 screens to study different molecular aspects of the fruit fly, Drosophila. Its efficacy in recovering FN in screens devoted to identifying viral HDFs in human systems is yet to be determined.

In general, these studies highlighted how using virus-host PPI databases to integrate the outcome of HT-RNAi data can maximize the relevance of the latter results, reducing FPs and FNs, increasing number of HDFs identified and eventually lead to identification of new drugs to combat viral infection.

A minor shortcoming of these studies has been that they have been largely biased towards a particular virus. For instance, all studies from[66,77-79] have been based on HIV screens while there has been only one meta-analysis on IFV screens[70]. It would be worthwhile and interesting for the community to see these approaches applied to other viruses. With the availability of several HT-RNAi screens for different viruses, a multi species meta-analysis can highlight similarities and differences between host-virus interactions, based on RNAi screen hits. Therefore, much effort still needs to be done to perform reliable HT-RNAi hits for a large number of viruses, including those such as hepatitis B virus, for which a reliable cell culture system is still not available.

Network properties of RNAi hits identified as viral HDFs

A complementary approach to integrate HT-RNAi with PPI datasets is to perform Network analyses. Particularly it is possible to characterize viral HDFs by computing several topological measures (network centralities) in addition to their biological function. These properties form the basis for interpreting the role of such hits from a network perspective. Furthermore, these scores also allow for a different level of hit prioritization for subsequent analysis. As mentioned earlier, specialized repositories host-pathogen interaction databases such as VirusMint, HIV-1-human protein interaction database, Host-Pathogen Interaction Database, VirHostNet, PHIDIAS, *etc.*[63-65,70,80,81] have fuelled these studies and shed new light on host-pathogen interactions. This section summarizes results from such studies and provides an overview of topological properties of viral HDFs.

In this context, the most comprehensive study has been recently published by Dyer *et al*[82]. The authors highlighted properties of host factors involved in the life cycle of 190 different pathogens from a network perspective. To this end, they collated experimentally identified human PPIs for 190 pathogen strains partitioned into 54 groups (35 viral, 17 bacterial, and two protozoan) pooled from 7 public databases[62,68,83-87], to determine properties of proteins targeted by most pathogens, including viruses. The main conclusion from this study was that pathogens preferentially target bottlenecks and hubs, implying that targeting central proteins is a common strategy shared by different pathogens. This study revealed that viral targeted host proteins also play a major role in different cancers of which some are induced by a viral infection itself (*e.g.*, Herpesvirus and Papillomavirus). Gulbahce *et al*[88] showed that the neighborhood of HDFs is as important as the HDFs themselves. They formulated what they term as "local impact hypothesis" wherein they propose that genes associated with virally implicated diseases are located in the neighborhood of viral targets. They tested their hypothesis by calculating the mean shortest path between genes that are viral targets and the ones implicated in a viral disease. This mean value was significantly shorter than between random samples. Scanning for genes within these path lengths, and subsequent experimental validation in human keratinocyte populations for HPV16 expressing E6, E7 proteins revealed 104 genes regulated by the 15 targets of E6 and E7 (these genes were 2 connections or paths away from these 15 targets). Of these 104 genes, 22 were also differentially expressed in IMR90 cells expressing HPV16 E6 or E7 proteins. A novel link was predicted between HPV and Fanconi anemia, through the E6->TP53->*FANCC* pathway through the *FANCC* gene which was one of the 22 genes described above.

Similar studies have been conducted recently with a specific focus towards HIV-1 host protein interactions. van Dijk *et al*[78] analyzed the HIV-1-Human protein interaction data and also highlighted the fact that viral products preferentially interact with host proteins that represent hubs or bottlenecks. Furthermore, they also determined enriched network motifs, statistically significant patterns of interacting proteins, from this network that allowed dynamic interpretation of interactions. For example, one of the enriched motifs included the 2 nodes feedback loop found in the HIV-host activation/inhibition network. This suggests the inhibitory nature of HIV proteins on human proteins that in turn inhibit the HIV protein. This motif occurred mostly for HIV Tat and Gp120 proteins with the human interferon γ. GO enrichment of the observed network motif indicated that it is involved in immune response.

Another independent study, performed using the same HIV-1-Human protein interaction dataset, reiterated that HIV-1 proteins attack hubs and bottlenecks over others[77]. By implementing an ascertainment bias, that normalizes weightage given to the genes based on publication count in order to avoid false interpretations[89], the authors came to two striking conclusions. First, HIV-1 interacting proteins and gene essentiality didn't have a strong correlation. Secondly, HIV-1 interacting proteins didn't tend to be disease-associated. Still, GO enrichment analyses of HIV-1 interacting proteins suggested that proviral and antiviral interactions are highly complex.

These studies thus have further enhanced our knowledge of the intricacies involved in HIV-1 infection, and opened new doors for the development of novel hypotheses.

Similar studies have been performed to experimentally determine the virus-host interactome of HCV, DENV and HTLV-1/2[90-92]. More recently a comprehensive study

focused on determining the interactome of 70 viral modulators of the innate immune response from 30 different viruses[93].

The common outcome of these studies is that viral proteins have the remarkable tendency to have significantly more targets, to be more central to the networks, to participate in more cellular pathways and are more likely to hold key positions in these pathways, as compared to an average human protein. On the same lines, both experimental and computational approaches helped identifying some common features of HDFs. These proteins have higher values of degree and of betweenness, which imply that viral proteins preferentially target proteins that are "central" to a given network. Smaller mean path length values of the HDFs, relative to the whole network also indicate that viral proteins target subnetworks that are "closely bound". Future studies in this direction might delve a bit deeper to uncover more topological features beyond what is already known.

Indeed, given the limited size of their genomes, viral products are required to interact with a high number of host proteins, which usually represent key factors regulating several biological processes. Morever these approaches can also help us to identify new HDFs: using these topological features, computational algorithms can be formulated to predict potential "generic" HDFs. For *e.g.*, PageRank centrality is one such feature. It is utilized by Google in order to decide the rank of the search hits. In the simplest sense, a node's importance is determined by the importance of its neighbors. Thus, the more "important" a node is topologically, the more it might also be biologically important, and therefore likely to be the target of viruses to overtake cellular functions. Jaeger *et al*[79], used this centrality measure to identify 21 surface membrane proteins critical for HIV-1 infection of which 11 are novel predictions, 3 are confirmed hits (chemokine receptor CCR1, chemokine binding protein 2 and duffy antigen chemokine receptor) and 7 have been confirmed in other studies. These receptors are potentially involved in different phases of HIV infection and influence progression of AIDS.

Degree, betweenness, pagerank and shortest-paths are just few of the many network centralities that have been defined to date for HDFs. It would be interesting to compute some additional measures to characterize HDFs. Quantifying structural properties of viral HDFs can help researchers in developing efficient machine learning algorithms to predict novel HDFs with greater accuracy. In addition to such predictions, a further, crucial layer of analysis would be to check for mouse orthologs of such predicted HDFs and verify if they are lethal for mouse. This step allows filtering of candidate HDFs, to be used for secondary validation, which can produce a lethal phenotype. Specificity and tissue localization of these HDFs can then be determined by utilizing tissue specific expression data from Protein Atlas (http://www.proteinatlas.org/)[94]. These steps would give a comprehensive overview of all HDFs beyond function and thus would aid in hypothesizing regulatory mechanisms and interactions between viral proteins and HDFs. Moreover, this would also reduce time, effort and cost of experimentalists and would serve as a guide to a more directed approach for hit validation.

All the above mentioned studies, both those considering RNAi hits and those which do not, strongly underlined the importance of inclusion of PPI network information to propose better hypotheses as well as therapeutic targets. They also highlighted the fact that for increasing reliability and confidence in HT-RNAi screens, validation by computational approaches *via* multiple data-types and sources is as important as verification with biological assays. Indeed, combination of data generated by different screens performed using the same virus, has evidently shown to strengthen the statistical significance of hits and reduce FP. The upcoming virus-host interaction databases, together with the availability of expression data and powerful, public tools for integrating and analyzing HT-RNAi screens will undoubtedly provide a comprehensive understanding of virus-host interactions at a cellular level.

CONCLUSION

Despite the remarkable efforts done so far to apply the use of HT-RNAi screening approaches to the study of the host cell-virus relationship, a great body of work is still required before we reach a comprehensive overview of how different viruses selectively exploit the host cell. This will finally lead to the design of specific antiviral compounds targeting host cell functions, which are therefore less prone to the selection of drug resistant viral strains. This process is strongly limited by the high number of different human pathogenic viruses, and that identification of HCFs required for viral replication necessarily relies on the availability of robust *in vitro* systems to propagate such viruses. Unfortunately, despite the tremendous advances made in the field, for example with the development of systems to propagate HCV[95-97], we are still lacking a system to efficiently propagate *in vitro* other important human pathogens (the most striking example being exemplified by Hepatitis B Virus, responsible for approximately 600 thousand causalities each year[98].

Beside this crucial shortcoming, it seems that the initial concerns related to the specificity and sensitivity of the HT-RNAi technology can be solved by combining data from different independent screens performed for the same virus, and by implementing sophisticated statistical algorithms that take into account differences within a cell population - an approach that have been proposed to strongly limit variance[54-56], as well as integrating HT-RNAi data with PPI datasets. In particular, the latter approach has been successfully used to reduce the number of FNs and FPs[75], to identify new HDFs[66], and also to identify new potential drug targets for treatment of viral infection[70]. Another major benefit of such integrative approaches relies in the possibility to perform network analysis of host factors and PPI datasets[77,82], thus en-

abling study the connections of viral products and the cellular effectors that are directly targeted by their action.

A third crucial point which needs to be considered is the growing need for in depth biochemical and biological characterization of the newly described hits. Indeed it is important not only to know the name and the molecular function of HDFs, but also the reason why exactly these factors are required for the life cycle of a given virus, for example, by enabling the formation of its replication compartments, or by being incorporated into the mature virion to mediate later on the recognition of a cellular receptor, to cite just a couple of examples of two well characterized viral HDFs for HCV, namely PIK4α and ApoE[34,60,99]. This knowledge enables at the same time to understand more in detail the mechanisms behind the usurpation of the host cell by viruses and to devise strategies to prevent this process.

In summary, progress still needs to be done in three directions before a complete understanding of the virus-host interplay: Development of appropriate cell culture systems to enable *in vitro* culture of human pathogenic viruses and their use to perform HT-RNAi screens, which should be rigorously analyzed by statistical analysis methods. Integration of data generated in different studies using the same virus, with other datasets, such as those deposited in PPI databases, to maximize sensitivity and specificity of the results. In depth characterization of identified hits of major relevance, including potential targets for the development of anti-viral drugs.

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TOPIC HIGHLIGHT

Gualtiero Alvisi, PhD, Assistant Professor, *Series Editor*

Architecture and biogenesis of plus-strand RNA virus replication factories

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Abstract

Plus-strand RNA virus replication occurs in tight association with cytoplasmic host cell membranes. Both, viral and cellular factors cooperatively generate distinct organelle-like structures, designated viral replication factories. This compartmentalization allows coordination of the different steps of the viral replication cycle, highly efficient genome replication and protection of the viral RNA from cellular defense mechanisms. Electron tomography studies conducted during the last couple of years revealed the three dimensional structure of numerous plus-strand RNA virus replication compartments and highlight morphological analogies between different virus families. Based on the morphology of virus-induced membrane rearrangements, we propose two separate subclasses: the invaginated vesicle/spherule type and the double membrane vesicle type. This review discusses common themes and distinct differences in the architecture of plus-strand RNA virus-induced membrane alterations and summarizes recent progress that has been made in understanding the complex interplay between viral and co-opted cellular factors in biogenesis and maintenance of plus-strand RNA virus replication factories.

Key words: Viral replication factory; Viral replication complex; Plus-strand RNA virus; Membrane remodeling; Virus-host interaction; Alphavirus; Enterovirus; Coronavirus; Flavivirus; Hepatitis C virus

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INTRODUCTION

As obligate intracellular parasites, all viruses depend on the host cell biosynthetic machinery in order to replicate their genome and generate progeny virus particles. A common feature among many different viruses is the induction of specialized membranous compartments, often forming organelle-like structures, within the cytoplasm of an infected cell. The unique features of those structures that facilitate virus propagation are best expressed by the commonly accepted term "viral replication factories". This review focuses on animal plus-strand RNA virus induced replication compartments. However, the concept of viral replication factories also applies to plant plus-strand RNA viruses as well as to other animal RNA viruses or some large DNA viruses such as vaccinia virus. These virus groups are covered by other reviews and will not be discussed here[1-3]. By analogy to an industrial manufacturing unit, virus induced replication factories serve multiple purposes.

To increase the local concentration of all necessary factors thus ensuring high efficacy

Local enrichment of the viral replication factors and co-

opted cellular proteins, together with all required metabolites, is the basis for highly efficient genome replication. This is achieved by reducing the three-dimensional (3-D) diffusion in the cytosol to two-dimensional diffusion on the surface of a membrane, thus increasing local reaction efficacy presumably by several orders of magnitude[4]. In fact, many replication enzymes encoded by plus-strand RNA viruses are membrane anchored as exemplified by the hepatitis C virus (HCV)[5]. In addition, also substrates such as ribonucleotides and metabolites providing energy for biosynthetic processes are enriched in replication compartments. Along these lines a recent study convincingly demonstrated locally elevated ATP levels at HCV replication sites[6].

To spatially coordinate different processes of the replication cycle such as RNA translation, replication and assembly

Compartmentalization allows the spatial separation and regulation of RNA translation, replication and packaging of the viral genome, thereby preventing an interference between these processes. Hence, ribosomes responsible for RNA translation and RNA-binding assembly factors are excluded from replication sites, thus avoiding interference with the replication machinery. Indeed, replication factories of all plus-strand RNA viruses are built up of ribosome-free membranes[7-11], and for all *Flaviviridae* (excess) amounts of core/capsid protein, binding RNA with high affinity, are frequently targeted to other cellular organelles such as lipid droplets (LDs) in the case of HCV and dengue virus (DENV)[12-14], or to the nucleus as observed for DENV[15,16] Japanese encephalitis virus[17] and West Nile virus (WNV)[18].

To create a protected environment shielding viral RNA and eventually also proteins from a hostile degradative environment

The generation of specialized membranous replication compartments protects viral replicase complexes and genomic RNA from degradation by cellular proteases or nucleases, respectively and "hides" the viral RNA genome from cytoplasmic sensors of the innate immune response. The RigI-like receptors efficiently recognize 5' triphosphorylated RNAs as well as double-stranded RNA (dsRNA) in a length-dependent manner[19,20], leading to mitochondrial antiviral signaling-mediated induction of interferons and nuclear factor κB-mediated inflammation[21]. Minimizing the exposure of stimuli to the innate immune surveillance, by the induction of innate sensor-protected organelle-like replication factories, is therefore an important evolutionary conserved feature of plus-strand RNA virus infection.

In the following we will summarize recent insights into the 3-D ultrastructure of plus-strand RNA virus-induced membrane rearrangements and discuss possible mechanisms of their biogenesis. Furthermore, viral subversion of host cell membrane biology, by interference with signaling pathways and recruitment of host cell factors contributing to biogenesis and maintenance of viral replication factories are highlighted.

MORPHOLOGY OF PLUS-STRAND RNA VIRUS REPLICATION FACTORIES

In the last few years, electron tomography has been instrumental to decipher the 3-D architecture of viral replication factories (for technical review see[22,23]). This accounts for evolutionary diverse plus-strand RNA viruses such as flock-house virus (FHV)[24], rubella virus (RUBV)[8], the two enteroviruses coxsackievirus B3 (CVB3)[25] and poliovirus (PV)[26], severe acute respiratory syndrome coronavirus (SARS-CoV)[11], equine arterivirus (EAV)[27], the two flaviviruses DENV[10] and WNV[9] and HCV[28]. Despite many differences in host range, virion morphology, genome organization, or donor membrane usage (Table 1), these analyses revealed that plus-strand RNA viruses appear to induce one of two different membrane alterations: the invaginated vesicle (InV) or spherule type and the double membrane vesicle (DMV) type. These morphologies that will be used in this review to group plus-strand RNA viruses might reflect the use of different host cell pathways and factors exploited by these viruses to establish the membranous replication compartment.

Architecture of replication factories corresponding to the InV/spherule type

Viral replication factories of the InV/spherule type are induced by alphaviruses such as Semliki Forrest virus (SFV)[29,30] and Sindbis virus[31], by FHV[24], RUBV[8], DENV[10] and WNV[9]. Although no 3-D reconstruction of alphavirus replication factories has been published yet, pioneering classical electron microscopy (EM) studies from Grimley *et al*[29] describing SFV replication sites at modified membranous structures, date back to the 1960s. Alphavirus infection induces so called cytoplasmic vacuoles (CPVs) (600-2000 nm in size), containing small invaginations called "spherules" with an average diameter of approximately 50 nm[29,32-34]. Surprisingly, at early time points after alphavirus infection, spherules are frequently found at the plasma membrane[30,31]. These spherules are subsequently internalized and become part of the endo-lysosomal membrane system, giving rise to CPVs. The single membrane invagination of spherules is continuous with its donor membrane and an approximately 8 nm small opening connects its interior with the cytoplasm[7]. Viral replicase proteins nsp1 to nsp4 as well as newly synthesized viral RNA localize to spherules[29,30]. Interestingly, the spherules themselves are devoid of ribosomes and viral capsid protein, which are frequently found juxtaposed to the spherule openings[7]. The first 3-D reconstruction of a plus-strand RNA virus replication factory was published by Kopek *et al*[24]. Electron tomography of FHV-infected cells revealed InVs on the outer mitochondrial membrane (OMM) (Figure 1A). Similar to alphavirus spherules, InVs found in FHV-infected cells are approximately 50 nm in di-

Table 1 Overview of plus-strand RNA viruses and induced replication factories

Invaginated vesicle/spherule type							
Superfamily/order	Alphavirus like	Alphavirus like	Alphavirus like	Alphavirus like			
Family	Togaviridae	Togaviridae	Togaviridae	Bromoviridae	Nodaviridae	Flaviviridae	Flaviviridae
Genus	Alphavirus	Alphavirus	Rubivirus	Bromovirus	Alphanodavirus	Flavivirus	Flavivirus
Species	SFV	SINV	RUBV	BMV	FHV	DENV	WNV
Genome	ss(+) RNA	ss(+) RNA	ss(+) RNA	tripartite ss(+) RNA	bipartite ss(+) RNA	ss(+) RNA	ss(+) RNA
Gene order	NS-S	NS-S	NS-S	NS-S	NS-S	S-NS	S-NS
Genome size (bases)	13000	12000	10000	8300	4500	10000	10000
Virion size (nm)	70	70	70	27	30	50	50
Envelope	Yes	Yes	Yes	No	No	Yes	Yes
Host	Mosquitoes, humans	Mosquitoes, humans	Humans	Plants, yeast	Insects	Mosquitoes, humans	Mosquitoes, mammals
Disease	Encephalitis	Sinbis fever	German measels	Plant disease	Unknown	Dengue fever	Mostly asymtomatic
Type of replication factories	Spherules at PM and CPVs	Spherules at PM and CPVs	Invaginations at CPVs	Spherules at the ER	Spherules at mitochondria	Invaginated vesicles at the ER	Invaginated vesicles at the ER
Diameter of invaginations (nm)	50	50	80-500	50-70	50	90	50-150
Primary membrane source	PM, endosomes	PM, endosomes	Endosomes	ER	Outer mitochondrial membrane	ER	ER
Proposed sites of replication	Inside spherules	Inside spherules	Inside invaginated vesicles	Inside spherules	Inside spherules	Inside invaginated vesicles	Inside invaginated vesicles
Double membrane vesicle type							
Superfamily/order		Picornavirales	Picornavirales	Nidovirales	Nidovirales	Nidovirales	
Family	Flaviviridae	Picornaviridae	Picornaviridae	Coronaviridae	Coronaviridae	Arteriviridae	
Genus	Hepacivirus	Enterovirus	Enterovirus	Coronavirus	Coronavirus	Arterivirus	
Species	HCV	PV	CVB3	SARS CoV	MHV	EAV	
Genome	ss(+) RNA	ss(+) RNA	ss(+) RNA	ss(+) RNA	ss(+) RNA	ss(+) RNA	
Gene order	S-NS	S-NS	S-NS	S-NS	S-NS	S-NS	
Genome size (bases)	9600	8000	8000	30000	30000	13000	
Virion size (nm)	50	30	30	80-160	80-160	40-60	
Envelope	Yes	No	No	Yes	Yes	Yes	
Host	Humans	Humans	Humans	Humans	Mice	Horses, donkeys	
Disease	Hepatitis	Poliomyelitis	HFM disease	SARS	HMM illness	Haemorragic fever	
Type of replication factories	DMVs	SMTs, DMVs	SMTs, DMVs	DMVs, VPs	DMVs	DMVs	
Length/diameter of replication factories	150 nm	100-200 nm, 100-300 nm	650 nm, 150 nm	150 nm, 1-5 μm	200-350 nm	90 nm	
Primary membrane source	ER	Golgi, ER	Golgi, ER	ER	ER	ER	
Proposed sites of replication	Possible role of DMVs but largely unknown	On SMT/DMV outer membrane	On SMT/DMV outer membrane	Inside DMVs	Inside DMVs	Inside DMVs	

NS: Genes encoding nonstructural proteins; S: Genes encoding structural proteins; ER: Endoplasmic reticulum; PM: Plasma membrane; HFM: Hand-foot-and mouth; SARS-CoV: Severe acute respiratory syndrome coronavirus; HMM: High mortality murine; DMVs: Double membrane vesicles; SMTs: Single membrane tubular vesicles; VPs: Vesicle packets; PV: Poliovirus; HCV: Hepatitis C virus; CVB3: Coxsackievirus B3; MHV: Mouse hepatitis virus; EAV: Equine arterivirus; CPV: Cytoplasmic vacuoles; RUBV: Rubella virus; SFV: Semliki Forrest virus; FHV: Flock-house virus; DENV: Dengue virus; WNV: West Nile virus; BMV: Brome mosaic virus; SINV: Sindbis virus.

ameter[35]. The single membrane building up the spherule is continuous with the OMM and a membranous neck with an interior diameter of approximately 10 nm connects the spherule lumen to the cytoplasm[24]. The sole viral replicase factor protein A as well as nascent viral RNA localize to spherules. By using high resolution electron tomography in combination with biochemical analyses, Kopek *et al*[24] determined that each spherule contains approximately 100 copies of protein A, covering the complete interior surface of the spherule and one or two copies of minus-strand RNA, the replication intermediate. RUBV induces replication factories with similar architecture. Replicase proteins p150 and p90, as well as nascent viral RNA localize to remodeled endosomes/lysosomes, termed cytopathic vacuoles[36-39]. Fontana *et al*[8] conducted electron tomography studies of RUBV replicon cells and observed spherule-like membrane invaginations. In addition, the authors detected bigger vacuolar invaginations and rigid straight membrane

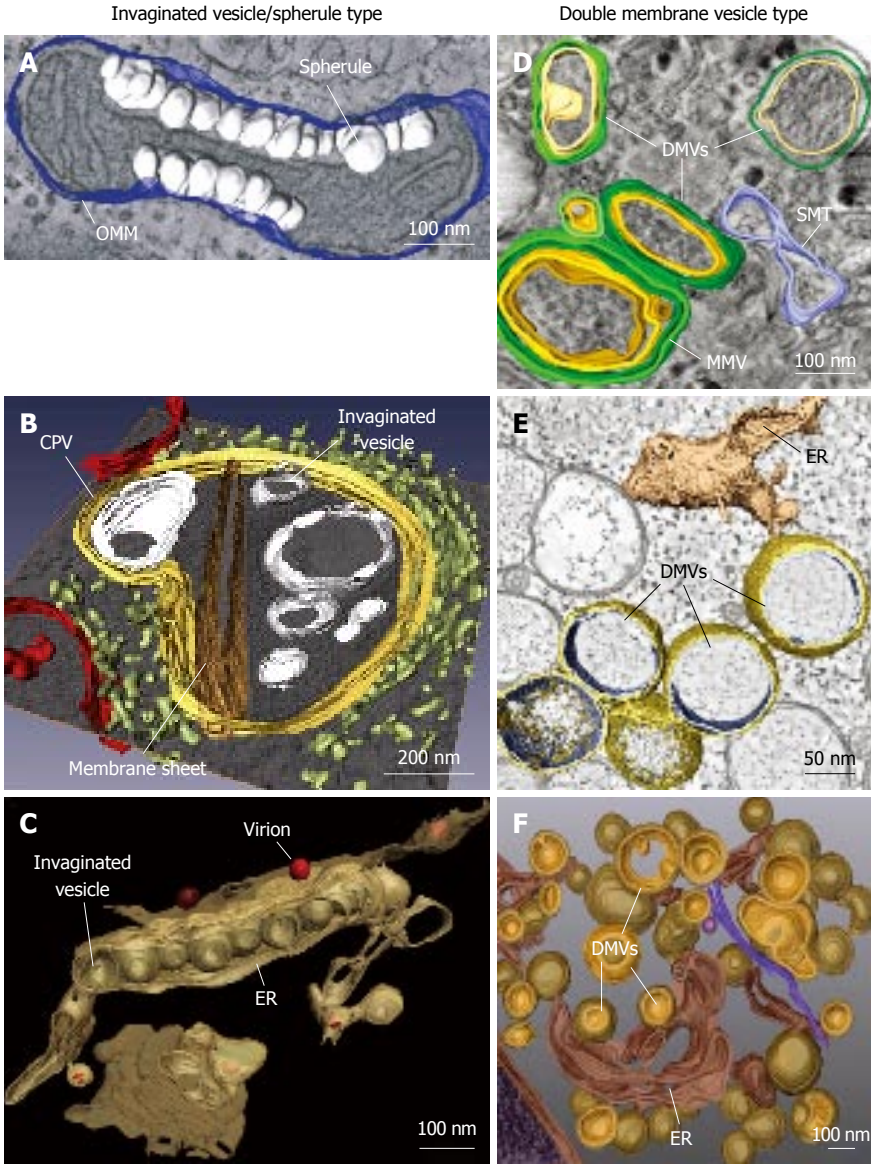

Figure 1 Classification and morphologies of plus-strand RNA virus-induced membrane alterations as revealed by electron tomography and three-dimensional reconstruction. Morphological similarities of membrane structures belonging to the invaginated vesicle (InV)/spherule type (A-C) or the DMV type (D-F). A: Flock-house virus (FHV); B: Rubella virus (RUBV); C: Dengue virus (DENV); D: Poliovirus (PV); E: Severe acute respiratory syndrome coronavirus (SARS-CoV); F: Hepatitis C virus (HCV). A: FHV-induced spherules (white) in the outer mitochondrial membrane (OMM; blue); B: InVs (white) and rigid membrane sheets (dark brown) in a modified endosome (the cytopathic vacuole, CPV) (yellow) in cells replicating RUBV. The rough endoplasmic reticulum (ER) is shown in light-green and mitochondria in red; C: Spherule-like invaginations (InVs) in the endoplasmic reticulum (ER) (brown) observed in DENV-infected cells. Newly formed progeny virions are shown in red; D: PV infection induces single membrane tubules (SMT) (blue), double membrane vesicles (DMVs) and multi membrane vesicles (MMVs). The DMV outer membrane is shown in green, the second membrane in yellow and a third membrane in case of MMVs in orange. These membranes are primarily derived from the Golgi; E: An interconnected network of ER-derived DMVs is found in SARS-CoV infected cells. The ER is colored in light-brown, interconnected outer membranes of DMVs in yellow and DMV inner membranes in blue; F: HCV-induced DMVs protruding from the ER (dark-brown). Outer membranes of DMVs are shown in light-brown and inner membranes in orange. Images are modified with permission from ©2007 Kopek *et al*[24] Plos Biol (A); ©2010 Elsevier Inc.[8] (B); ©2009 Elsevier Inc.[10] (C); © 2012 American Society for Microbiology[26] (D); ©2008 Knoops *et al*[11] Plos Biol (E); ©2012 Romero-Brey *et al*[28] Plos Pathog (F).

sheets, which are continuous with the outer membrane of the CPV and whose interior is connected to the cytoplasm (Figure 1B). Mitochondria localized in close proximity to CPVs and were probably engaged in supplying energy for viral replication. Importantly, CPV membranes were free of ribosomes, but frequently surrounded by rough ER sheets and Golgi stacks, thus facilitating translation and encapsidation of viral RNA, respectively[8,40]. In contrast to the replication compartments of these plus-strand RNA viruses, replication factories of Flaviviridae, exemplified by WNV and DENV, are most likely derived from the ER. Apart from the rather undefined convoluted membranes (CMs) found in DENV-infected cells, our laboratory revealed by means of electron tomography vesicular invaginations of approximately 90 nm diameter in the rough ER, which are connected by approximately 10 nm diameter necked channel openings to the cytoplasm[10] (Figure 1C). Likewise, Gillespie *et al*[9] observed in WNV replicon-containing cells, InVs in the rough ER, which were connected *via* neck-like membranous pores to the

cytoplasmic space. For both viruses, replicase proteins and dsRNA, assumed to represent a replication intermediate, localized to InVs[10,41], whereas ribosomes were excluded from the InVs, but localized in close proximity on ER membranes[9,10]. Interestingly, DENV virion budding was observed frequently close to or directly opposite of pore-like vesicle openings[10], highlighting the spatial orchestration of replication and assembly steps in the DENV replication cycle.

Architecture of DMV-like replication factories

DMV-like replication factories are formed by the enteroviruses CVB3[25] and PV[26], SARS-CoV[11], EAV[27] and HCV[28]. For PV, probably the best studied plus-strand RNA virus, structural alterations of cellular membranes were observed already more than 50 years ago. They were first described as membranous vesicles of heterogeneous size and termed clear vacuoles and U-bodies[42]. Recent electron tomography studies of PV-infected cells by Belov *et al*[26] revealed apparently empty and heterogeneous single membrane-branching tubular structures (100-200 nm diameter) that were detected at very early time points after infection. As infection progresses, these tubular structures developed into DMVs (100-300 nm in diameter), filled with presumably cytoplasmic material (Figure 1D). The very early membrane alterations were derived from the Golgi[26], but PV-induced vesicles were earlier shown to "bud" from the ER in a COP Ⅱ -dependent manner and to contain lysosomal markers[43,44]. Replicase proteins 2C and 3A as well as nascent viral RNA localize to the outside of both single and double membrane structures[26,44]. A similar 3-D architecture of virus-induced membrane alterations was recently described for CVB3, another member of the Picornaviridae family. By using electron tomography, Limpens *et al*[25] observed interconnected single membrane tubular structures (approximately 650 nm in length and approximately 80 nm in diameter) at the onset of infection. These structures transformed into DMVs (approximately 160 nm in diameter) and multimembranous structures by an enwrapping process. Immunofluorescence analyses showed co-localization of CVB3 replicase proteins 3A and 3D with dsRNA[25] during the log phase of viral replication, when predominantly single membrane tubules were present in infected cells. However, the exact localization of viral proteins and RNA with respect to the rearranged membranes remains to be determined. In contrast to picornavirus-infected cells, Knoops *et al*[11] revealed that early after infection SARS-CoV replication factories appear as presumably ER-derived CMs and interconnected DMVs (250 nm in diameter) (Figure 1E), which subsequently merge into vesicle packets (VPs) (1-5 μm in size), most likely by fusion of the DMV outer membranes. Electron tomography analysis showed that SARS-CoV-induced DMVs do not exist as isolated structures, but their outer membrane is continuous to other DMVs, CMs, or the rER, thus explaining the transition from DMVs to VPs[11]. Viral replicase proteins nsp3, 5 and 8 were sporadically found in DMVs, whereas the majority localized to CMs. In contrast, dsRNA a marker for viral replication intermediates, was predominantly found inside DMVs or inside the vesicles of VPs[11]. The DMV interior was devoid of ribosomes, which were found on the outer membranes of DMVs or rER sheets in close proximity to DMVs. Virus budding was detected at the outer membrane of VPs, arguing for spatial organization of the different steps of the SARS-CoV replication cycle[11]. A comparable architecture of virus-induced membrane rearrangements was observed for EAV, a SARS-CoV related virus that also belongs to the order *Nidovirales*. By means of electron tomography, Knoops *et al*[27] unraveled a reticulovesicular network of interconnected DMVs in EAV-infected cells. However, in contrast to SARS-CoV induced DMVs, those observed for EAV were smaller in diameter (approximately 90 nm), but also exhibited continuous outer DMV membranes. Replicase proteins nsp3 and 9 associated with DMVs and surrounding membranes and as in case of SARS-CoV, dsRNA was prominently found inside DMVs[27]. In addition to the reticulovesicular DMV network, membrane tubules with approximately 43 nm diameter were associated with the EAV capsid protein N and frequently found in close proximity to DMVs[27], thus providing another example for the spatial coordination of replication and assembly steps. Although belonging to the *Flaviviridae* that includes DENV and WNV, HCV-induced viral replication factories are very different from the InV/spherule type, but more comparable to those of unrelated *Picornaviridae* or *Coronaviridae*. Electron tomography of HCV-infected cells conducted in our laboratory, revealed ER-derived DMVs as the predominant structures, which partially develop into MMVs at later stages of infection[28] (Figure 1F). HCV-induced DMVs have an average diameter of approximately 150 nm. Around 50% of DMVs were linked to the ER *via* the outer membrane giving rise to a neck-like structure, but later on DMVs seem to detach from their donor membrane. Only a small subset (approximately 10%) exhibited a pore-like opening to the cytoplasm, whereas the vast majority showed "sealed" inner and outer membranes.

By using correlative-light-EM, DMVs were shown to contain the viral replicase protein non-structural protein (NS)5A, which co-localizes with dsRNA as shown by fluorescence microscopy[28]. However, direct detection of the HCV replication site at the ultrastructural level was not successful. DMVs localized in close proximity to the rER and LDs, an important organelle for HCV assembly[13], arguing for a compartmentalization of viral RNA translation, replication and assembly steps[28].

TOPOLOGY OF PLUS-STRAND RNA VIRUS REPLICATION SITES

In order to identify viral replication sites, various methods have been applied to detect viral RNA at the ultrastructural level. Initial studies employed metabolic radiolabeling of nascent viral RNA with ^{3}H-labeled uridine or adenosine and subsequent EM-based detection by autoradiography using film emulsions[29,33]. Others applied

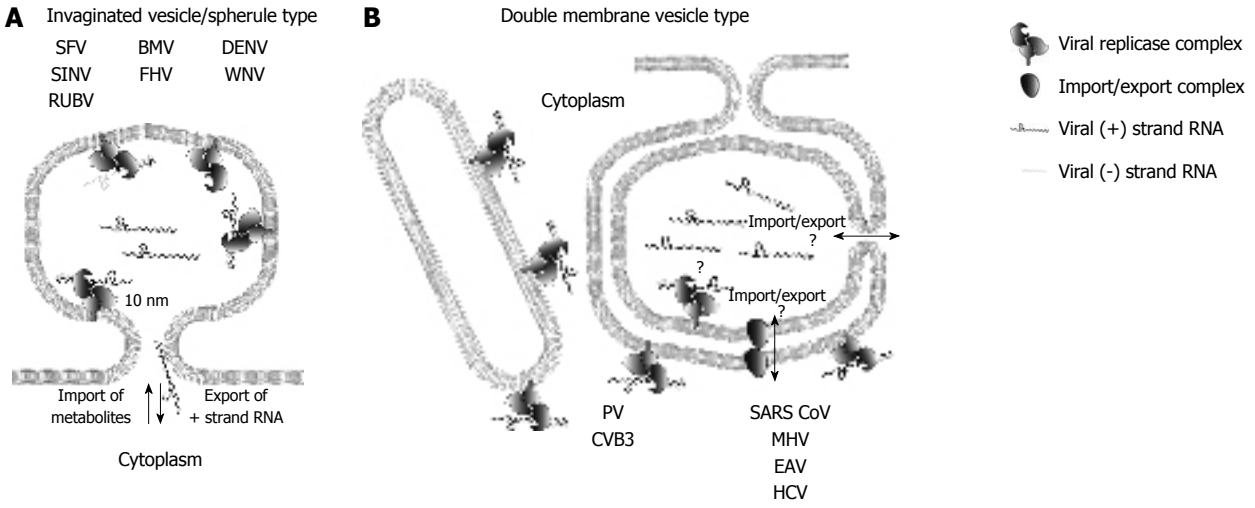

Figure 2 Topology of plus-strand RNA virus replication sites. A: RNA replication of plus-strand RNA viruses inducing invaginated vesicles/spherules is thought to occur in the spherule lumen. Examples of viruses inducing this type of remodeled membranes are denoted. The neck like opening to the cytoplasm allows export of progeny RNA destined for translation or packaging, as well as import of metabolites required for RNA replication; B: Possible topologies of RNA replication site for double membrane vesicle (DMV)-type factories. Enterovirus replication is thought to take place on the outer membrane of single membrane tubules and DMVs, whereas the exact replication site of nidovirales and hepatitis C virus (HCV) is still unclear as indicated by the question marks. Replication in the DMV lumen would require import and export across two membranes, which could be mediated *via* a proteinacous transport complex, or *via* an opening to the cytosol of not yet fully closed DMVs (see main text for details). SFV: Semliki Forrest virus; SINV: Sindbis virus; RUBV: Rubella virus; BMV: Brome mosaic virus; FHV: Flock-house virus; DENV: Dengue virus; WNV: West Nile virus; PV: Poliovirus; CVB3: Coxsackievirus B3; SARS-CoV: Severe acute respiratory syndrome coronavirus; MHV: Mouse hepatitis virus; EAV: Equine arterivirus.

in situ hybridization methods by using nucleic acid probes and detection *via* colloidal gold particles[45], labeling of dsRNA replication intermediates by using a dsRNA-specific antibody[10,11,27,41], or antibody-based detection of bromouridine, incorporated into nascent viral RNA[24,26,30]. Importantly, for identification of viral replication sites the detection of metabolically labeled viral RNA is the only reliable method; hybridization techniques do not discriminate between replicating RNA or RNA used *e.g.*, for translation or virion formation. The same applies to detection of viral RNA with the dsRNA-specific antibody that can bind to RNA structures present in genomes of plus-strand RNA viruses, thus excluding specificity for detection of active viral RNA replication. Indeed, by using the incorporation of ethynyl-uridine (EU) into actively replicating coronavirus RNA and subsequent detection *via* click-chemistry, Hagemeijer *et al*[46] observed a loss of overlap of the EU- signal with dsRNA labeling during the course of infection. For replication factories of the InV/spherule type it is commonly accepted that viral replicase complexes reside on the invaginated membrane and thus, RNA replication takes place in the spherule lumen[9,10,24,30,36,41,47] (Figure 2A). The neck-like connection to the cytoplasm allows import of all required metabolites (*e.g.*, ribonucleotides) and export of newly synthesized RNA destined for translation or packaging into the capsid. By using bromouridine labeling of nascent viral RNA, this assumption has been confirmed for RUBV[36] alphaviruses[30], FHV[24] and brome mosaic virus (BMV)[47]. However, identification of flavivirus replication sites (DENV and WNV) up to now relies only on the immuno-detection of dsRNA[10,41]. DMV-type replication factories exhibit a topologically more complex situation, as both membranes of the DMV are often sealed and no connection to the cytosol is obvious[11,25-28]. Hence, for this group of plus-strand RNA viruses different topologies of replication sites are currently proposed (Figure 2B). In case of enteroviruses, replication is thought to occur on the cytoplasmic side of single and double-membrane structures[26,48]. However, the fact that during the log phase of viral replication predominantly single membrane tubules are found in PV-or CVB3-infected cells[25,26] suggests that active enteroviral replicase complexes primarily reside on these membrane structures. In this case one might speculate that RNA replication ceases after membrane enwrapping and DMV sealing, but this remains to be determined. In case of SARS-CoV and EAV, the bulk of viral RNA is found inside the lumen of DMVs as determined by dsRNA labeling[11,27]. Moreover, nascent EAV and mouse hepatitis virus (MHV) RNA is associated with DMV membranes as shown by bromouridine labeling[49,50] arguing that DMVs are sites of viral replication. However, if replication takes place inside the DMV lumen, it is unclear how import of metabolites and export of viral RNA occurs. Even with high resolution electron tomography, neck-like membrane discontinuities of approximately 10 nm, as observed for InV/spherule like replication factories, have not been found[11,27]. Thus, proteinaceous channels or transporters might be responsible for linking DMV interior to the cytosol. In fact, proteins such as the ER translocon complex or the mitochondrial transporter outer membrane and transporter inner membrane complexes mediating transport across one or two membranes, respectively (reviewed in[51,52]), have not been visualized by electron tomography so far. Thus, an up to now unidentified enzymatic function of

viral proteins containing multiple transmembrane passages and/or recruitment of a cellular factor executing this transport function is in principle possible.

Another hypothesis is discussed for HCV-induced DMVs, which accumulate during the log phase of viral RNA replication[28]. Only a small fraction of DMVs has an opening to the cytoplasm, whereas the majority exhibits entirely closed membranes. It is speculated that replication might occur in the interior of DMVs as long as they are linked to the cytosol, but upon membrane sealing DMVs would contain dead end replication complexes that are no longer active. Apart from this possibility, the other models described above also apply to HCV, but further studies are required to define the exact site of RNA replication.

MECHANISMS OF MEMBRANE ALTERATIONS INDUCED BY VIRAL PROTEINS

Given the highly complex architecture of plus-strand RNA virus replication factories on one hand and the small genetic coding capacity of these viruses on the other hand, it is obvious that membrane alterations are induced by the concerted action of viral and cellular factors. In this section we will discuss the intrinsic membrane-active properties of viral proteins; host cell factors contributing to formation of replication factories are summarized in section 6 of this article. In principle, membrane bending can be achieved by (1) local alterations of membrane lipid composition; or (2) asymmetric interaction of proteins with membranes (Figure 3). The latter includes scaffolding of the membrane by peripheral proteins, insertion of asymmetric proteins or protein complexes into the membrane, insertion of amphipathic helices into one leaflet of the lipid bilayer or interactions of the membrane with the cytoskeleton (reviewed in[53-55]) (Figure 3C-H). Negative membrane curvature is predominant for the InV/spherule like replication factories (Figure 3A), with spherules bending the membrane away from the cytoplasm, whereas DMV-like replication factories exhibit positive membrane curvature, *i.e.*, the outer DMV membrane is bent towards the cytosol (Figure 3B). However, regions of positive membrane curvature are also present in neck like openings of spherules (Figure 3A) and the inner membranes of DMVs exhibit negative membrane curvature (Figure 3B), respectively, highlighting the complexity of membrane bending events in the induction of plus-strand RNA virus replication factories.

Several viral proteins were identified that play crucial roles in the induction of membrane rearrangements. For instance, the membrane-associated protein nsp1 of alphaviruses interacts with anionic phospholipids *via* an amphipathic helix (AH) (Figure 3G)[56] and is additionally tethered to membranes *via* a palmitic acid residue in the carboxy-terminal region of the protein[57]. AH-mediated nsp1 membrane association proved crucial for SFV replication[58], whereas palmitoylation mutants could be rescued by second site compensatory muations *in vitro*[59] but are attenuated *in vivo*[60]. Nonetheless, only expression of the P123 protein precursor induced spherule-like structures as seen in SFV-infected cells[61], demonstrating the need of nsp1 to recruit and interact with other viral and/or cellular proteins to induce spherule-like membrane rearrangements. The FHV replicase factor protein A exhibits binding affinity to specific anionic phospholipids, namely phosphatidic acid, phosphatidylglycerol and cardiolipin[62], which are enriched in mitochondrial membranes. Protein A is inserted into the OMM by an N-terminal mitochondria-targeting transmembrane sequence[63]. Self-interaction of protein A is required for FHV replication and mediated by multiple domains (Figure 3F)[64]. Nevertheless, protein A is not sufficient to induce spherule like membrane invaginations in the OMM, which requires in addition a replication-competent template RNA and protein A's polymerase activity[65]. Interestingly, these requirements do not apply to the FHV-related plant virus BMV, for which expression of protein 1a is sufficient to induce spherule-like membrane invaginations[47]. All flaviviral proteins are associated directly or indirectly with membranes either by amphipathic helices or transmembrane domains or both (Figure 3E-G). Fully processed DENV and WNV NS4A is sufficient to induce rearrangements of the ER membrane[66,67], although membrane structures induced by sole NS4A expression are morphologically distinct from those induced in infected cells. Studies of the membrane topology of DENV NS4A revealed an ER luminal helix in the central region of the protein, lying in plane in the luminal membrane leaflet[66]. This topology is compatible with the negative curvature of InVs, arguing that NS4A increases the surface area of the ER membrane on the luminal side. However, the role of NS4A self-interaction and a possible interplay with other viral (*e.g.*, NS4B) and cellular factors, in membrane remodeling remain to be determined. Also for HCV that in contrast to DENV and WNV induces DMV-type replication factories, membrane remodeling activity has been attributed to viral proteins, most prominently to NS4B. It has been proposed that expression of this protein is sufficient to induce the membranous HCV replication compartment (the "membranous web")[68]. NS4B is a highly hydrophobic protein that contains at least four transmembrane passages[69]. It is palmitoylated at its C-terminus[70] and in addition contains multiple AHs in its N-and C-terminal domains[71-73] (Figure 3E-G). The membrane-associated C-terminal domain of NS4B or the AH within it have been reported to alter membrane integrity (Figure 3G)[74,75], corroborating membrane remodeling activity of NS4B. In addition, NS4B self-interacts in a homo- and heterotypic fashion[76], which is required for HCV RNA replication[76,77] (Figure 3F). Interestingly, mutations in NS4B that inhibit HCV RNA replication also alter NS4B self-interaction and morphology of HCV-induced DMVs[77], implying a functional relationship between DMV morphology and HCV RNA

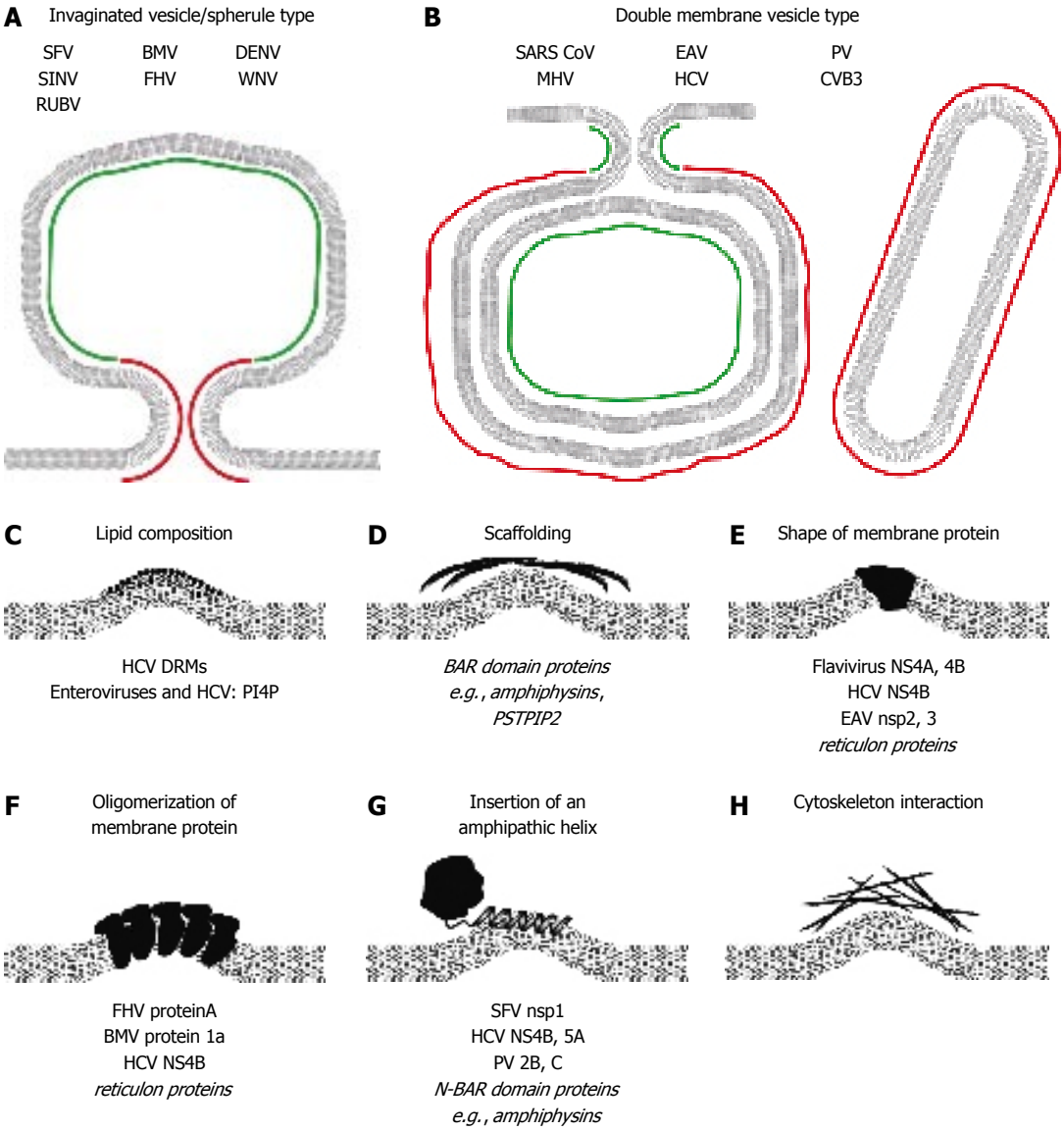

Figure 3 Mechanisms responsible for induction of membrane curvature likely contributing to formation of plus-strand RNA virus replication factories. Positive and negative membrane curvature for invaginated vesicle/spherule type (A) and double membrane vesicle type (B) replication factories is indicated in red and green, respectively. Mechanisms of membrane modifications/bending are schematically depicted in (C-H). Examples of viral and cellular (in italics) proteins are given at the bottom of each panel (see main text for details). SFV: Semliki Forrest virus; SINV: Sindbis virus; RUBV: Rubella virus; BMV: Brome mosaic virus; FHV: Flock-house virus; DENV: Dengue virus; WNV: West Nile virus; SARS-CoV: Severe acute respiratory syndrome coronavirus; MHV: Mouse hepatitis virus; EAV: Equine arterivirus; HCV: Hepatitis C virus; PV: Poliovirus; CVB3: Coxsackievirus B3; DRM: Detergent-resistant-membrane; BAR: Bin-Amphiphysin-Rvs; PSTPIP2: Proline-serine-threonine phosphatase interacting protein 2; NS4A: Non-structural protein 4A; NS4B: Non-structural protein 4B; N-BAR: N-terminal amphipathic helix containing BAR; PI4P: Phosphatidylinositol-4-phosphate.

replication. Inspite of the membrane remodeling activity of NS4B, we recently found that only an NS3-5A polyprotein fragment is sufficient to induce DMV structures with morphological similarity to those observed in HCV-infected cells[28]. In fact, MMVs and a small number of DMVs were observed in cells over-expressing just NS5A, which is a dimeric and eventually oligomeric replicase factor with an N-terminal AH[78-82] (Figure 3G). Based on these observations we assume that a concerted action of HCV replicase factors is required for the biogenesis of the membranous web. Similar observations have been made for *Nidovirales*. For instance, coronavirus nsp3, 4 and 6 contain multiple membrane-spanning domains[83-85]. These proteins appear to play a central role in membrane remodeling (Figure 3E), because mutations *e.g.*, in nsp4 exhibit impaired viral replication and altered DMV morphology[86]. Nsp3 of the related EAV also contains multiple transmembrane segments and is another key player in the induction of DMVs. Expression of nsp2-3 suffices to induce DMVs[87] and their morphology is dramatically altered by nsp3 mutations affecting an ER luminal loop[88].

In case of enteroviruses, proteins 2B, 2C or 3A are membrane-associated *via* transmembrane passages or AHs[89-91] and the expression of 2BC either alone[92] or in concert with 3A[93] induces structures similar to those in infected cells. Interestingly, expression of only 2C induces more dramatic membrane remodeling including

tubular membrane swirls inside a highly dilated ER[92], implying a regulatory role of 2B in 2C membrane remodeling. Taken together these data suggest that membrane-associated proteins of plus-strand RNA viruses have intrinsic membrane remodeling properties. However, in most cases more than one viral factor, eventually in concert with recruited cellular proteins are involved in generation of viral replication factories. Further studies using *e.g.*, recombinant viral proteins and model membranes are required to elucidate the intrinsic membrane remodeling properties of these viral proteins.

POSSIBLE ROLE OF AUTOPHAGY IN THE FORMATION OF DMV LIKE REPLICATION FACTORIES

Autophagy is an evolutionary conserved catabolic mechanism for degradation of long-lived organelles and cytoplasmic material and is crucial for cell homeostasis (reviewed in[94]). Virus-host co-evolution has shaped multiple mechanisms involving autophagy that either promote or restrict viral replication (reviewed in[95]). Due to morphological similarities between DMV-like replication factories and the also double-membrane nature of autophagosomes, it has been suggested that autophagy plays a role in biogenesis of viral replication compartments of this type as induced by enteroviruses[44,96,97], coronaviruses[98,99] and HCV[100,101]. Indeed, lipidation of microtubule - associated protein 1 light chain 3 (LC3), a key event in the induction of autophagy, was observed after enterovirus infection[96,97,102] or over-expression of 2BC[102]. Interestingly, knock-down of central autophagy components or pharmacological inhibition of the pathway decreased viral replication only slightly, whereas generation of progeny virus was clearly reduced arguing that autophagy contributes primarily to virus assembly and release[103]. In case of PV, electron tomography studies of infected cells revealed primarily single membrane structures during the log phase of viral replication, which subsequently developed into DMVs by collapsing and/or enwrapping events[26]. These data argue against a central role of autophagy *per se* in PV replication, although some factors of this pathway might contribute to heterogeneity of vesicular structures at later stages of infection. Similar observations have been made for the *Coronaviridae*. MHV infection induces lipidation of LC3 that co-localizes with nsp2/3 in infected cells[99]. Moreover, the non-lipidated form of LC3 is prominently found on isolated membrane fractions from MHV-infected cells, together with components (*e.g.*, EDEM1) of the ER-associated degradation pathway[99]. However, functional studies based on knockouts of central autophagy factors led to contradictory results. In one study, ATG5 knockout decreased virus propagation dramatically[98], whereas others found that MHV replication is independent of ATG5[104] or ATG7[99]. Hence, a central role of the complete autophagy pathway in generation of coronavirus DMVs seems unlikely, although some single factors such as LC3 might add to it. Conflicting observations concerning a role of autophagy in virus propagation have also been made in case of HCV. One study claims that autophagy is implicated in translation of incoming viral RNA, but is dispensable for RNA replication[105]. Other studies suggest that autophagosomes are sites of active replication[101] or promote assembly and release of progeny virus[106]. In addition, a recent study showed an autophagy-mediated down-regulation of innate immune response[107]. In this case, knock-down of autophagy components reduces HCV RNA replication due to a stronger innate immune response. The reasons for these discrepant results are not known, but might be due, at least in part, to the use of different Huh7 cell clones, which are known to differ in their capacity to mount innate antiviral defenses. Although LC3 lipidation is observed upon HCV infection[105] and the protein is associated with HCV membrane fractions[100], EM-based studies of membrane remodeling events argues for a role of autophagy in formation of MMVs eventually as part of a cellular stress response induced by massive membrane alterations[28]. Taken together, the role of autophagy in biogenesis of DMV like replication factories remains rather elusive. Single components of the conventional cellular autophagy are possibly involved, whereas a direct contribution of the complete pathway in generation of DMV replication compartments is rather unlikely. Finally, autophagy might be an epiphenomenom, being activated as a cellular stress response to tremendous amounts of virus induced cytoplasmic membrane alterations, engaged in cell homeostasis and survival during viral infection.

PLUS-STRAND RNA VIRUS SUBVERSION OF CELLULAR MEMBRANE BIOLOGY

Since plus-strand RNA viruses induce massive remodeling of cytoplasmic membranes, but most often have very limited genetic coding capacity it is not surprising that these viruses utilize membrane-active host cell factors and exploit cellular pathways involved in membrane homeostasis.

Viral utilization of co-opted membrane-active proteins of the host cell

Enteroviruses and the plant virus BMV co-opt cellular reticulon proteins, which are required for membrane remodeling and viral RNA replication[108,109]. The evolutionary conserved reticulon protein family is characterized by a common reticulon homology domain (RHD) involved in shaping the ER by inducing and stabilizing highly curved ER tubules[110,111]. Morphogenic properties can be attributed to elongated hydrophobic, partially membrane-spanning hairpin structures within the RHD[112], which in concert with its oligomerization properties[113] increases the surface on the cytoplasmic membrane leaflet, thereby inducing positive curvature (Figure 3E and F). Reticulon 3 has been shown to directly interact with the enterovirus 2C protein[108] and is thus likely engaged in induction and/or stabilization of positive membrane curvature of

enterovirus replication factories. BMV replicase protein 1a directly binds to and recruits reticulon proteins to spherule-like membrane invaginations and might stabilize positive membrane curvature in neck-like openings to the cytoplasm or facilitate expansion of the spherule volume by partially neutralizing overall negative membrane curvature[109]. It remains to be determined whether reticulon proteins are co-opted also by other plus-strand RNA viruses, especially those deriving their replication compartments from the ER. Another example of cellular membrane-shaping proteins are amphiphysins that are involved in formation of alphavirus replication factories[114]. These Bin-Amphiphysin-Rvs (BAR) domain containing proteins play pivotal roles in endocytosis and intracellular membrane trafficking (reviewed in[115]). Structural analyses revealed dimerization of the BAR domain mediated by helical coiled-coil interactions[116], giving rise to a concave banana-shaped structure. This complex has a positively charged inner surface, which interacts with negatively charged membrane phospholipids (reviewed in[117]). Thus, BAR domain-containing proteins sense and stabilize membrane curvature by scaffolding mechanisms, to which in case of N-terminal amphipathic helix containing BAR proteins membrane insertion of N-terminal amphipathic helices contributes (Figure 3D and G). Alphavirus nsp3 binds to SH3 domains in amphiphysin *via* a proline-rich sequence and mediates the recruitment to viral replication factories. Knock-down experiments proved an important role of amphiphysins in alphavirus replication[114]. Although their contribution to membrane remodeling during alphavirus infection remains to be discovered, one could envisage a similar mechanism as proposed for BMV and reticulon proteins. Proline-serine-threonine phosphatase interacting protein 2 (PSTPIP2) also belongs to the BAR protein superfamily (Figure 3D) and was recently shown to be involved in membrane alterations induced by HCV[118]. PSTPIP2 binds to NS4B and NS5A and thereby is recruited to the membranous replication compartment[118]. In fact, knockdown experiments showed the crucial role of PSTPIP2 in HCV membrane remodeling and RNA replication[118]. Interestingly, upon over-expression PSTPIP2 induces cytoplasmic tubular membranes[118], highlighting its ability to induce positive membrane curvature. Hence, PSTPIP2 is probably engaged in inducing and/or stabilizing positive membrane curvature of HCV replication factories. Another mechanism by which viruses can rearrange intracellular membranes has become evident for enteroviruses. PV appears to hijack components of the cellular secretory pathway, explaining why PV replication is sensitive to brefeldin A (BFA) treatment[119]. Viral proteins 3A and 3CD recruit the ADP ribosylation factor (Arf)-GEFs (GTP exchange factors) Golgi-specific BFA-resistance guanine nucleotide-exchange factor 1 (GBF1) and BFA-inhibited guanine nucleotide-exchange protein 1/2 (BIG1/2) to replication sites, leading to elevated levels of activated Arf-GTP in infected cells[120]. Arf proteins are central regulators of membrane dynamics and vesicle budding in the secretory pathway. Upon activation by Arf-GEFs, the GTP-bound form exerts its function on target membranes by recruitment of effector proteins such as lipid modifying enzymes and coat complexes, thus facilitating vesicle budding (reviewed in[121,122]). GBF1-mediated Arf activation is crucial for PV replication, although typical membrane alterations can be observed in BFA-treated cells that over-express viral proteins[123]. Nevertheless, PV replication factories of BFA-treated cells were shown to be inactive, implying a functional difference of remodeled membranes independent from morphological abnormalities[123]. Hence, PV subversion of cellular Arf-GEFs is important for maintenance of functional replication factories, rather than primary membrane remodeling. In summary, plus-strand RNA viruses recruit and utilize membrane-active host cell proteins in order to generate and/or maintain replication factories. Donor membrane usage defines engaged cellular factors, as enteroviruses employ Arf-GEFs from the Golgi, alphaviruses subvert amphiphysins localized at the plasma membrane and enteroviruses and BMV utilize ER-resident reticulon proteins (Figure 4).

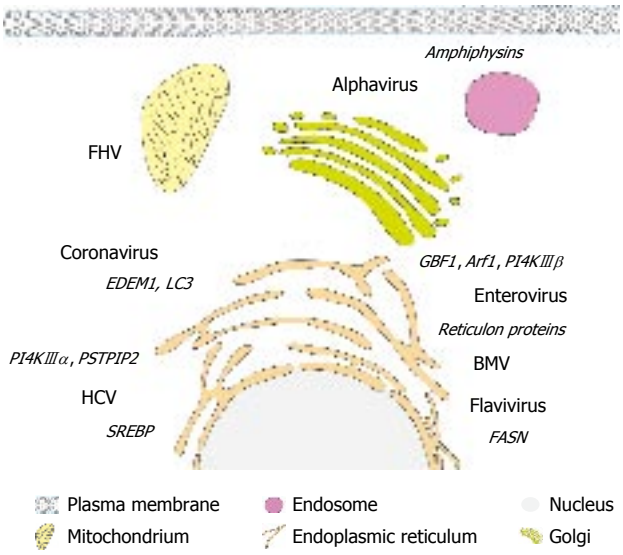

Figure 4 Primary membrane source and host cell factors subverted by different plus-strand RNA viruses. A eukaryotic cell and (endo)membrane organelles are depicted schematically as indicated on the bottom. Viruses are displayed next to their primary membrane source organelle and recruited host cell factors involved in lipogenesis and membrane remodeling are denoted in italics adjacent to each virus name. FHV: Flock-house virus; PSTPIP2: Proline-serine-threonine phosphatase interacting protein 2; HCV: Hepatitis C virus; SREBP: Sterol regulatory element-binding protein; PI4K: Phosphatidylinositol-4-kinase; GBF1: Golgi-specific BFA-resistance guanine nucleotide-exchange factor 1; BMV: Brome mosaic virus; FASN: Fatty acid synthase.

Viral exploitation of pathways regulating homeostasis of cellular membranes

Apart from remodeling existing intracellular membranes, virus infection often induces *de novo* lipid and membrane biosynthesis in order to increase membrane surface area, which is required for the formation of viral replication factories. Indeed, alteration of cellular lipid homeostasis and virus-induced lipogenesis has been reported for a broad range of plus-strand RNA viruses, including

FHV[124], BMV[125], RUBV[126], DENV[127,128], WNV[129], enteroviruses[130] and HCV[131-133]. FHV mainly stimulates glycerophospholipid metabolism[124] and inhibition of phospholipid synthesis leads to destabilization of protein A and decreases viral replication[134]. In case of DENV, high-throughput analysis revealed multiple changes in the cellular lipidome of infected cells, including specific up or down regulation of main structural lipids species, highlighting the link to virus-induced membrane remodeling[128]. Additionally, DENV NS3 recruits fatty acid synthase (FASN), which catalyzes the rate limiting step in lipid biosynthesis at the sites of viral replication[127]. Thus, DENV appears to subvert FASN for *de novo* lipid synthesis in order to generate new membranes for the formation of viral replication factories. This feature is also observed for the closely related flavivirus WNV[129], stressing the impact of this particular virus host interaction to generate flavivirus replication factories.

HCV induces lipogenesis *via* the sterol regulatory element-binding protein (SREBP) pathway[131]. SREBPs are major transcription factors for expression of genes required for lipid biosynthesis. SREBPs reside as inactive membrane-bound precursors in the ER, which upon stimulation traffic to the Golgi. There they are proteolytically activated by site 1 protease (S1P) and S2P and subsequently stimulate gene transcription (reviewed in[135]). Proteolytic cleavage of SREBP and transactivating phosphorylation has been observed in HCV-infected cells or upon over-expression of NS4B, leading to elevated levels of transcripts involved in lipogenesis such as FASN[131,136]. However, by using S1P-specific inhibitors SREBP-mediated lipogenesis was found to be dispensable for HCV replication but required for assembly and release of progeny virus[137]. In addition, the HCV replicase complex was shown to reside in detergent-resistant membranes[138]. These membrane micro domains, designated lipid rafts, are enriched for cholesterol, sphingolipids and certain proteins and they form nanoscale-ordered protein-lipid assemblies (reviewed in[139]). Sphingolipid synthesis is stimulated upon and required for HCV replication[140] and it was shown that the NS5B RdRP is activated by sphingomyelin in a genotype-specific manner[141]. A shared feature of plus-strand RNA viruses inducing DMV-like replication factories is their dependence on members of the phosphatidylinositol-4-kinase (PI4K) family and their product, phosphatidylinositol-4-phosphate (PI4P). Both enteroviruses and HCV rely on PI4P for functional replication factories, which is generated by recruitment of PI4KⅢβ in case of enteroviruses[130] and PI4KⅢα in case of HCV[142]. In non-infected cells PI4P localizes to the Golgi and the inner leaflet of the plasma membrane, where it fulfills important functions by providing "signatures" to distinct membrane compartments and by recruiting multiple factors involved in vesicle budding and lipid biosynthesis[143,144]. Subversion of PI4KⅢβ by enteroviruses is executed *via* 3A-GBF1 interaction, activating Arf that in turn recruits PI4KⅢβ to viral replication factories[130]. Locally elevated PI4P levels allow specific binding of the viral 3D RdRp to the membrane favoring viral replication. Knock-down as well as pharmacological inhibition of PI4KⅢβ dramatically decreases enteroviral replication[130].

In case of HCV, the replicase proteins NS5A and NS5B directly interact with PI4KⅢα, which is thereby recruited to HCV replication sites[142]. Importantly, in HCV-infected cells PI4P that is usually enriched at the Golgi and the plasma membrane, is prominently enriched at the ER-derived sites of HCV replication[142,145]. Numerous siRNA screens identified PI4KⅢα as a major host dependency factor for HCV replication[142,146-149], and pharmacological inhibition of PI4KⅢα activity efficiently blocks viral replication[145]. In the absence of PI4KⅢα, expression of HCV proteins NS3-5B still induces DMVs, but these vesicles are smaller in diameter, very homogeneous and tend to cluster[142]. This morphological change of the overall structure of the membranous web correlates with impaired replication although DMV morphology *per se* is only moderately affected. This phenomenon is reminiscent to what has been shown for BFA-treated cells over-expressing PV proteins[123]. These findings argue for a role of PI4P in recruiting viral factors such as PV 3D RdRp or so far unidentified cellular proteins, rather than acting as a structural lipid building up membranous replication factories. Importantly, the closely HCV-related flaviviruses DENV and WNV, which form replication factories of the InV/sperule type, do not depend on PI4KⅢα/β or PI4P[129,142], highlighting a functional relationship between PI4P and DMV-like replication factories. Therefore it would be very interesting to investigate the dependence of EAV and coronavirus replication on PI4P. We note that for the latter PI4KⅢβ was shown to be required for virus entry[150].

Taken together, the strong dependence of positive strand RNA viruses on cellular membrane-active proteins and on pathways implicated in cellular membrane homeostasis, renders those host cells factors very attractive targets for future antiviral drug development.

CONCLUSION

Although important discoveries on the 3D architecture of plus-strand RNA virus replication factories have been made, current knowledge is largely descriptive and important information about mechanisms is missing. For instance, the exact topology of RNA replication sites for DMV-type replication factories is elusive. Identification of these sites will require novel experimental techniques such as metabolic labeling of nascent viral RNA and its visualization by using microscopy methods with high resolution and specificity. Likewise, membrane remodeling events responsible for the biogenesis of replication factories are mostly unknown. They are probably mediated by a complex interplay of viral and cellular factors, but precise contributions of individual factors and their temporal and spatial coordination remain to be discovered. Studying the impact of single proteins or combinations

thereof on model membranes *in vitro* or on membranes in cellulo by using correlative light-EM based methods as described recently for studies of membrane remodeling events during endocytosis[151] are possible ways to address this topic. Furthermore, determining the proteome and lipidome of purified viral replication factories will shed light on viral and host cell factors involved in biogenesis and activity of these membranous compartments. This approach has been used with great success for small intracellular vesicles such as COP I vesicles or neuronal transport vesicles[152,153] providing insight into individual membranous structures with unprecedented detail. Another emerging field is the specific in-membrane interaction of proteins with certain lipids, as recently shown for COP I machinery protein p24 and the sphingolipid SM 18 being implicated in regulation of COP I vesicle budding[154]. The tight membrane association of proteins of plus-strand RNA viruses suggests that such specific protein-lipid interactions also occur for this large virus group. Finally, time-resolved (imaging) techniques might shed light onto coupling of viral RNA translation and replication as recently shown for HCV[155] and furthermore onto transport processes of viral and cellular components inside and outside replication factories. These studies will be instrumental to integrate their functional role into the complete viral replication cycle.

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TOPIC HIGHLIGHT

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Innate host responses to West Nile virus: Implications for central nervous system immunopathology

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Abstract

West Nile virus (WNV) is an emerging neurotropic flavivirus that has recently spread to America and Southern Europe *via* an enzootic/epizootic bird-mosquito-bird transmission cycle. The virus can occasionally infect humans through mosquito bites, and man-to-man transmission has also been reported *via* infected blood or organ donation. In the human host, WNV causes asymptomatic infection in about 70%-80% of cases, while < 1% of clinical cases progress to severe neuroinvasive disease; long-term neurological sequelae are common in more than 50% of these severe cases. The pathogenesis of the neuroinvasive form of WNV infection remains incompletely understood, and risk factors for developing severe clinical illness are largely unknown. The innate immune response plays a major role in the control of WNV replication, which is supported by the fact that the virus has developed numerous mechanisms to escape the control of antiviral interferons. However, exaggerated inflammatory responses lead to pathology, mainly involving the central nervous system. This brief review presents the salient features of innate host responses, WNV immunoevasion strategies, and WNV-induced immunopathology.

Key words: West Nile virus infection; Innate immunity; Antigen presenting cells; Inflammation; Interferon and cytokines; Central nervous system

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INTRODUCTION

West Nile virus (WNV) is a lipid-enveloped virus that contains a single stranded, positive sense RNA genome. The virus is introduced into the host by an infected vector (mosquitoes generally belonging to the genus *Culex*) during its blood meal. WNV was originally found in Africa and in the Middle East but has recently reached America[1,2], where it has spread throughout the United States. In the last 15 years, WNV has also caused several human outbreaks in southern Europe[3-5].

Most individuals infected with WNV remain asymptomatic. In 20%-30% of cases, WNV causes a mild flu-

like illness. In such cases, symptoms appear suddenly and may include malaise, eye pain, headache, myalgia, gastrointestinal discomfort and rash[6]. Less than 1% of infected individuals develop neurological symptoms, including aseptic meningitis, febrile convulsion in children, encephalitis or myelitis, the last of which causes acute flaccid paralysis[7-9]. Long-term neurological sequelae are common in more than 50% of neuroinvasive cases. The virus can infect neurons in areas as diverse as the cerebral cortex, basal ganglia and thalami, as well as the brainstem and cerebellum. Currently, risk factors for developing severe clinical illness are unknown. However, it is clear that WNV central nervous system (CNS) disease occurs with increased frequency in immunocompromised individuals and the elderly[10-12].

Innate immune responses are believed to be crucial for the control of WNV replication, as a potent and rapid type I interferon (IFN) response is essential for the successful control of WNV infection in mice[13]. As a first line of defence, the host cell senses the presence of the virus by pathogen recognition receptors (PRRs), such as toll-like receptors (TLRs) and retinoic acid-inducible gene (RIG)- I like receptors. Binding of viral components to these receptors activates adaptor proteins, which in turn activate transcription factors, and induces a release of soluble mediators, including type I IFNs[14,15]. Members of RIG- I like receptor family (RIG- I and melanoma differentiation-associated gene, MDA5) and TLR family (TLR3 and TLR7) are the major innate host sensors of WNV infection. RIG- I and MDA5 are cytosolic RNA helicases that recognize ssRNA and dsRNA. RIG- I and MDA5 transmit their signal through a common adaptor molecule, IFN-promoter stimulator (IPS)-1, thus activating transcription factors such as IFN regulatory factor (IRF) 3 and IRF7 to induce the transcription of type I IFN and antiviral genes. TLR3 and TLR7 are expressed primarily in endosomes and are activated by dsRNA and ssRNA, respectively. Engagement of TLR7 leads to the activation of a signalling pathway involving an intracellular adaptor protein, myeloid differentiation primary response gene 88 (MyD88), the activation of IRF7 and the induction of type I IFNs. TLR3 activates the adaptor molecule TIR-domain-containing adapter-inducing IFN-β and induces alternative pathways that lead to the activation of the transcription factors IRF3 and nuclear factor κB (NF-κB), which consequently induce type I IFNs and inflammatory cytokines, respectively.

Antigen presenting cells (APCs) are among the first cells that encounter the virus after infection; WNV is injected intradermally by a mosquito bite and most likely initially replicates in Langerhans dendritic cells (DCs). The infected Langerhans cells migrate to draining lymph nodes from which the virus enters the bloodstream[16]. Primary viremia disseminates the virus to the reticuloendothelial system (macrophagic cells), where replication further augments viremia (secondary viremia), followed by spread in various organs including the brain. Monocytes and polymorphonuclear leukocytes (PMNLs) are readily recruited and activated following infection in rodent models[17].

Thus, cells of the innate immune system and their receptors are the first to encounter WNV after infection in the host, and the interaction between the virus and factors of innate immunity likely determines the outcome of the infection. In addition, macrophages (Mφs) constitute an important fraction on the inflammatory infiltrate observed in the CNS of WNV infected patients[18], suggesting that cells of innate immunity can also contribute to immunopathology in the course of WNV infection.

INTERPLAY BETWEEN CELLS OF INNATE IMMUNITY AND WNV

Despite the potentially critical role of APCs during WNV infection, few studies have addressed the effect of WNV infection on APCs obtained from humans. Human myeloid DCs (mDCs) have been shown to be among the targets of WNV infection. Production of tumor necrosis factor (TNF)-α and IFN-α in infected mDCs requires viral replication[19,20] (Figure 1). Conversely, plasmacytoid DCs (pDCs) are resistant to infection but are clearly activated upon contact with the virus through stimulation of endosomal TLRs. Upon activation with WNV, pDCs release higher amount of IFN-α than mDCs[19]. It has been demonstrated that glycosylated strains of WNV use DC-SIGN (a C-type lectin that binds high-mannose N-linked glycans present on the surface of viral glycoproteins) as an attachment receptor to bind mDCs, leading to enhanced infection in cell cultures[20]. This finding suggests that glycosylated strains of WNV, mainly belonging to lineage I, exhibit an increased capability to infect mDCs and thus higher pathogenicity.

Human monocytes and monocyte-derived Mφs also undergo productive infection upon *in vitro* incubation with WNV[21]. Interestingly, these cells are infected without gross cytopathic effects, suggesting that they possess effective defence mechanisms against WNV[22]. The lack of cell deterioration upon WNV infection in monocytes/Mφs also suggests that these cells play a significant role as a reservoir in initial (or secondary) viral replication and dissemination. Upon WNV infection, monocyte-derived Mφs release interleukin (IL)-8, IFN-α, IFN-β and TNF-α[22,23]. However, in Mφ cultures activated by LPS and IFN-γ, WNV infection down-modulates the secretion of IL-1β and IFN-β and inhibits the JAK/STAT signalling pathway[23], as a potential strategy employed by the virus to evade the host response (see below).

Notably, monocyte-derived Mφs from elderly individuals show increased susceptibility to WNV infection and augmented expression levels of TLR3 upon infection, as compared to young subjects. Once stimulated with the virus, cells from the elderly also secrete higher levels of IFN-β and IL-6[24]. This *in vitro* model of WNV infection suggests that the age-associated impairment of the innate immune response to WNV may contribute to increased severity of this viral infection in older individuals.

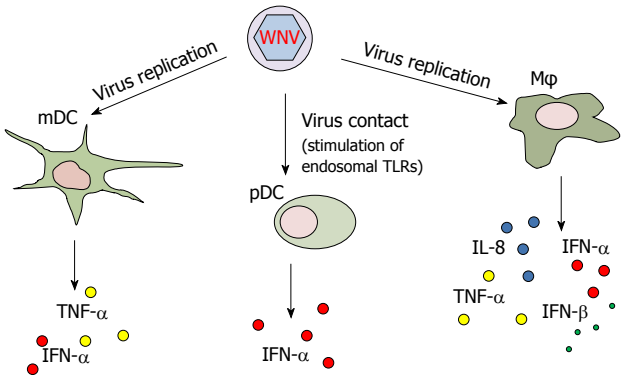

Figure 1 West Nile virus stimulates the production of interferons and pro-inflammatory cytokines in human antigen presenting cells. TLR: Toll-like receptors; WNV: West Nile virus; mDC: Myeloid dendritic cells; pDC: Plasmacytoid dendritic cells; Mφ: Macrophages; TNF: Tumor necrosis factor; IFN: Interferon; IL: Interleukin.

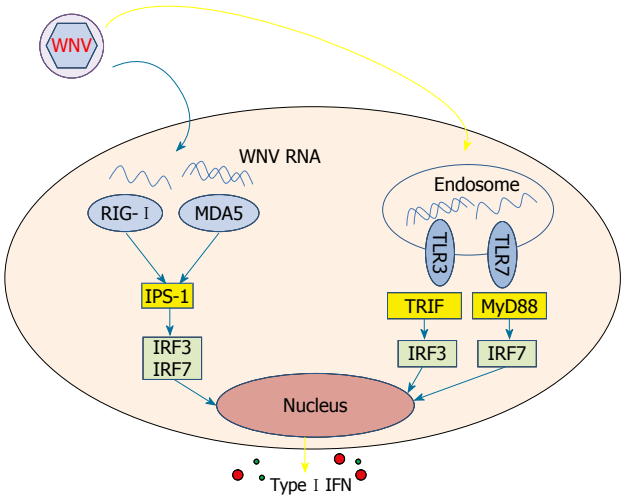

Figure 2 Receptors of innate immunity, adaptors and transcription factors involved in recognition of West Nile virus. WNV: West Nile virus; IFN: Interferon; IPS-1: IFN-β promoter stimulation-1; IRF: IFN regulatory factor; MDA5: Melanoma differentiation-associated protein 5; MyD88: Myeloid differentiation primary response gene 88; RIG; Retinoic acid-inducible gene; TLR: Toll-like receptor; TRIF: TIR-domain-containing adapter-inducing interferon-β.

IFNs AND WNV

Type I IFNs represent a major innate immune control and comprise various IFN-α and one IFN-β, which are secreted by leukocytes and parenchymal cells during viral infections[25]. These cytokines induce an antiviral state by up-regulating genes with direct and indirect antiviral functions. Type I IFNs also link innate and adaptive immunity by inducing DC maturation and by directly activating B and T cells[26].

As mentioned in the previous section, human mDCs, pDCs and monocyte-derived Mφs secrete type I IFNs upon contact with WNV[19-21]. The role of these antiviral mediators upon WNV infection and the role of the pathways involved in IFN secretion have been elucidated only in animal models. Studies in mice indicate that type I IFNs play a crucial role in the early control of WNV infection. Mice lacking IFN-α/β receptor are highly vulnerable to WNV, and uncontrolled viral replication occurs with rapid dissemination to the CNS and 100% mortality[13]. In addition, it has been observed that pre-treatment or treatment with type I IFNs *in vitro* inhibits WNV replication in Vero cells[27,28]. Additionally, treatment of primary murine neurons *in vitro* with IFN-β either before or after infection increased neuronal survival independent of its effect on WNV replication[13]. Altogether, these findings in animals and *in vitro* cultured cells support a crucial role for type I IFNs in the early phases of WNV infection by preventing viral replication and protecting infected neurons from death.

Cells recognize WNV and respond by producing type I IFNs through the endosomal receptors TLR3 and TLR7, thus activating the adaptor MyD88 and transcription factors IRF3 and IRF7 (Figure 2). This response has been demonstrated in rodent models of infection, as mice with genetic defects in any one of these receptors[29,30], adaptor[31] or transcription factors[32,33] have a higher mortality rate with experimental WNV infection (reviewed in[34]).

WNV RNA can also induce the release of type I IFNs by triggering RIG- I , which appears to be involved in the early phases of response to the virus[35]. MDA5, belonging to the RIG- I receptor family, is also involved in sensing WNV RNA; abrogation of both RIG- I and MDA5 pathways blocks activation of the antiviral response to WNV, while such an effect is not as evident if only one of the two pathways is ablated[36]. In line with these findings, infected mice lacking IPS-1, the central adaptor for RIG- I and MDA5, display uncontrolled inflammation that is coupled with the failure to protect against WNV infection[37]. Thus, TLR3 and TLR7, as well as RIG- I and MDA5, are activated by WNV and appear to induce redundant IFN-mediated responses that trigger downstream effective adaptive responses.

The regulation of IFN responses could be more complex than indicated by the present understanding. Increasing evidence indicates a crucial antiviral role for the inflammasome, a cytoplasmic multi-protein complex that recruits inflammatory caspases and triggers their activation[38]. For example, recent evidence shows that caspase-12, an important component of the inflammasome signalling, plays an important role in WNV infection by influencing RIG- I activity and type I IFN release[39]. The role of other inflammasome complex proteins in influencing the release of type I IFNs during WNV infection has not been investigated.

IFN type Ⅱ, *i.e.*, IFN-γ, is mainly produced by CD8+ T cells, it is also secreted by γδ T cells and natural killer cells and may contribute to innate immune control of viral infections. *In vivo*, IFN-γ restricts early WNV dissemination to the CNS; mice deficient in either IFN-γ or the IFN-γ receptor show a higher peripheral viral load, augmented entry into the CNS and increased lethality[40,41]. Notably, no major deficits of adaptive immunity were found in these studies, suggesting that IFN-γ plays mainly an early innate role in the control of WNV infection.

In recent years, a third type of IFN has been described. Originally termed IL-28a/b and IL-29, these proteins have been re-classified as IFN-λs, based on the similar modes of induction and the antiviral activities that they share with the type I and type Ⅱ IFNs[25]. In support to their antiviral role, IFN-λ3 has recently been identified as key cytokine in the control of a flavivirus infection, *i.e.*, hepatitis C virus[42]. The role of these mediators during the course of other flaviviruses is relatively unknown; only one study has examined the role of IFN-λ in the control of WNV to date. Similar to type I IFN, IFN-λ prevents infection by WNV virus-like particles in susceptible cells but fails to inhibit viral replication in cells infected prior to the addition of this cytokine[43].

INHIBITION OF IFN-INDUCED RESPONSES BY WNV

WNV has successfully evolved countermeasures to overcome host innate immunity and productively infect host cells by using a combination of two strategies: (1) passive evasion of the interaction with cellular PRRs and/or (2) active inhibition of different steps of the intracellular pathways that lead to type I IFN production and signalling.

Passive evasion of PRR activation

WNV may regulate the time of induction of the host cell antiviral response by modulating the activation of IRF3 during early phases of infection. WNV does not actively inhibit the RIG- I pathway but rather delays IRF3 activation, possibly by preventing host cells from sensing viral replication shortly after infection[35,44], thus allowing the virus to replicate to high titers before the host cells can mount an effective antiviral response.

Active inhibition of type I IFN production and signalling

WNV attenuates type I IFN response by targeting multiple steps of the induction and signalling cascade, and a number of nonstructural viral proteins (NSs), such as NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 have been implicated in this process[45,46]. WNV NS1, a protein secreted from infected cells, inhibits TLR3 signalling by preventing IRF3 and NF-κB nuclear translocation[47], and NS2A has been identified as an inhibitor of IFN-β gene transcription[48].

WNV also targets essential elements of the IFN signalling pathway and thus prevent the induction of antiviral genes. Type I IFN signalling initiates when IFNα/β bind to type I receptors (IFNRs) on the cell surface. This process results in the activation of JAK1 and Tyk2, phosphorylating STAT1 and STAT2, which, in association with IRF9, form a heterotrimeric complex known as IFN-stimulated gene (ISG) factor 3. This complex translocates to the nucleus where it induces hundreds of ISGs. The expression of WNV NSs prevents the accumulation of IFNR1 in multiple cells through a non-canonical protein degradation pathway, contributing to the inhibition of the IFN response[49].

The NS5 codified by the virulent lineage I strains of WNV can function as an efficient IFN antagonist by preventing the phosphorylation and nuclear translocation of STAT1[50], while NS4B inhibits JAK1 and TyK2 phosphorylation thus blocking the STAT1 and STAT2 signalling cascade and the subsequent ISG expression. WNV infection actively promotes a redistribution of cholesterol within the cells, which contributes to the down-regulation of the IFN-stimulated JAK-STAT antiviral response to infection and thus facilitates viral replication and survival[51]. Furthermore, characteristic membranous structures induced during WNV replication are connected to viral immune evasion mechanisms, providing partial protection from the IFN-induced antiviral protein MxA[52].

However, viral control of the IFN signalling cascade is not complete, as demonstrated by occurrence of IFNα/β induction and ISG expression during WNV infection. WNV may attenuate or modulate the innate antiviral response, and the ability of only pathogenic lineage I WNV isolates to inhibit the JAK/STAT signalling pathways indicates the importance of this fine modulation as a feature of WNV pathogenesis[53].

ROLE OF INNATE IMMUNITY IN THE PATHOGENESIS OF THE NEUROINVASIVE FORM OF WNV INFECTION

Despite its severity, the pathogenesis of the neuroinvasive form of WNV infection remains incompletely understood. Knowledge in this field relies almost completely on studies in murine models, while the roles of innate mechanisms in inducing protection or causing pathology in human WNV disease are still poorly known. The increased risk of severe WNV infections for immunosuppressed patients[12,54] and the successful infection outcome in a transplant recipient by the modulation of the immunosuppressive regimen[55] suggest that an intact immune system is essential for the control of WNV infection. On the other hand, it is generally recognized that a major hallmark of WNV pathogenesis is neuroinflammation[56,57], which is caused by exaggerated innate and acquired immune responses.

WNV is believed to first multiply in mDCs and monocytes/Mφs before spreading to the brain[58], and recent evidence indicates that early viral replication in myeloid APCs has a crucial pathogenetic role; silencing such replication in Mφs and mDCs effectively suppresses virus-induced encephalitis in mice[59]. Mechanisms underlying this clear-cut effect could rely on (1) an increased viral burden induced by infected APCs, which would be sufficient for the virus to cross the blood-brain barrier, or (2) WNV-infected Mφs acting as "Trojan horses" to carry the virus into the brain[60]. Accumulation of inflammatory monocytes into the brain and their differentiation to Mφs and microglia can also worsen neuroinflammation and CNS injury, as demonstrated in a murine model of non-lethal WNV infection[61]. As an additional pathogenetic

role of infected APCs, recognition of WNV nucleic acid in monocytes/microglia by TLR3 leads to the production of TNF-α, which results in a loss of tight junctions, allowing the entry of WNV and immune cells into the perivascular space of the brain in mice[56]. Further, increased levels of macrophage migratory inhibitory factor (MIF) (a potent pro-inflammatory mediator and chemotactic factor that is produced by activated Mφs) have been found in the serum and CSF of WNV-infected patients, and abrogation of MIF in WNV-infected mice mitigates clinical disease by inducing a remarkably reduced number of infiltrating WNV-infected leukocytes in the CNS[62]. Thus, activation of cells of the monocyte/Mφ system by WNV appears to result in important neuropathological consequences, and exaggerated innate responses may cause inflammation, altering the blood brain barrier permeability and allowing the virus to enter the CNS.

On the other hand, early monocytosis induced by WNV in a murine model of infection appears to be protective against lethal disease[63]. Further murine studies on WNV infection indicate a protective role for Mφs[64] and for TLR3, the latter being essential for restricting WNV replication in neurons and protecting the host from lethal encephalitis[29]. Finally, CCR5, a chemokine receptor expressed on Mφs and T cells, is a critical antiviral agent and survival determinant in WNV infection in mice that acts by regulating the trafficking of leukocytes to the infected brain[65]. These controversial studies suggest that monocyte/Mφ involvement and TLR simulation may contribute to inducing protection or causing immunopathology during WNV neuroinvasive disease in mice.

In addition to monocytes/Mφs, other cells belonging to the innate immune system may contribute to the pathogenesis of neuroinvasive WNV infection. For example, PMNLs predominate in the CSF of patients with WNV meningitis and encephalitis in 40% of cases[8] and are recruited shortly after infection into the CNS in an experimental model of WNV infection[17]. In infected mice, the expression of PMNL-recruiting chemokines was dramatically elevated in early phases after infection and PMNLs were quickly recruited to sites of WNV infection. Depletion of PMNLs prior to WNV challenge paradoxically lowered viremia and enhanced survival[66], suggesting that these cells have a pathogenic role in the early phases of WNV infection. Mechanisms that underlie the contribution of PMNLs to the pathogenesis of WNV infection may include the efficient replication of WNV in PMNLs; these cells may act as a virus reservoir, as PMNLs are the predominant cell type recruited to the site of infection and carry the highest amount of virus[66].

As part of the innate response, two important cell types within the CNS respond to infection, *i.e.*, microglia and astrocytes. These cells have been found to be infected in tissue sections from patients with WNV meningoencephalitis[67]. WNV-infected human astrocytes are capable of releasing matrix metalloproteinase 1, 3 and 9, which contribute by disrupting the blood brain barrier and degrading tight junction proteins[68].

In addition to glial cells, which are classically considered to be the predominant source of pro-inflammatory mediators in the CNS during WNV infection, WNV-infected neurons release pro-inflammatory mediators, contributing to neuronal cell death and glial cell activation[69]. Additionally, pro-inflammatory chemokines, such as IFN-γ inducible protein 10, monocyte chemoattractant protein-5 and monokine induced by IFN-γ, are important triggers of inflammation in the brain, and their early up-regulation in the CNS is followed by the up-regulation of TNF-α at the same site in a rodent model of WNV infection[57]. Further, treatment of infected neuronal cells with antibodies blocking TNF-α and other pro-inflammatory mediators results in a significant reduction of WNV-mediated neuronal death[69], suggesting that such mediators play a major role in the pathogenesis of WNV infection in the CNS.

However, pro-inflammatory factors also possess a crucial role in defence against WNV, and leukocyte trafficking into the brain induced by TNF-α protects mice against lethal infection[70]. Altogether, contradictory findings regarding the role of innate responses to WNV infection in mice have been reported; early responses appear to be beneficial or harmful depending on the model. Different experimental settings, including the virus passage history, virus inoculation route and dose, time between the infection and the experiments and potential diverse inflammatory response to WNV in different murine strains, may account for these contradictory findings. Early control of WNV by innate responses would likely effectively restrict WNV dissemination, while continuous triggering and/or excessive reactivity of innate receptors to the virus may contribute to enhanced inflammation, which is known to be a main contributor to WNV neuropathology as a result of CNS invasion.

CONCLUSION

The innate immune response is considered to be a major controller of WNV replication, a notion that is also supported by the fact that the virus has developed numerous mechanisms to escape the control of antiviral IFNs. However, exaggerated innate immune responses appear to be detrimental and lead to neuropathology. Importantly, the role of aging in enhancing the WNV-induced innate immune response has recently been clarified in an *in vitro* model of infection[24]. Nevertheless, the mechanisms triggering protection or pathology during natural WNV infection are largely unclear.

The interplay between WNV and innate responses has been mainly studied in animal models, while studies on the effect of WNV on human cells of innate immunity are restricted to *in vitro* cultured cells. All of the abovementioned models have a common limitation, *i.e.*, the transmission of the virus does not occur by a typical route. This limitation leads to two major biases: (1) a lack of transmission of saliva and potential symbionts with the mosquito bite, and (2) a lack of "natural" stimulation

of Langerhans DCs and/or antimicrobial peptides at the inoculation site. Thus, further immunological studies in individuals undergoing natural infection are required to better understand the immunopathogenesis of WNV disease, as elucidating the immunopathological mechanisms is essential to inform novel approaches to combat this infection.

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TOPIC HIGHLIGHT

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Paramyxovirus evasion of innate immunity: Diverse strategies for common targets

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Abstract

The paramyxoviruses are a family of > 30 viruses that variously infect humans, other mammals and fish to cause diverse outcomes, ranging from asymptomatic to lethal disease, with the zoonotic paramyxoviruses Nipah and Hendra showing up to 70% case-fatality rate in humans. The capacity to evade host immunity is central to viral infection, and paramyxoviruses have evolved multiple strategies to overcome the host interferon (IFN)-mediated innate immune response through the activity of their IFN-antagonist proteins. Although paramyxovirus IFN antagonists generally target common factors of the IFN system, including melanoma differentiation associated factor 5, retinoic acid-inducible gene-I, signal transducers and activators of transcription (STAT)1 and STAT2, and IFN regulatory factor 3, the mechanisms of antagonism show remarkable diversity between different genera and even individual members of the same genus; the reasons for this diversity, however, are not currently understood. Here, we review the IFN antagonism strategies of paramyxoviruses, highlighting mechanistic differences observed between individual species and genera. We also discuss potential sources of this diversity, including biological differences in the host and/or tissue specificity of different paramyxoviruses, and potential effects of experimental approaches that have largely relied on *in vitro* systems. Importantly, recent studies using recombinant virus systems and animal infection models are beginning to clarify the importance of certain mechanisms of IFN antagonism to *in vivo* infections, providing important indications not only of their critical importance to virulence, but also of their potential targeting for new therapeutic/vaccine approaches.

Key words: Paramyxoviridae; Innate immunity; Signal transducers and activators of transcription 1; Signal transducers and activators of transcription 2; Melanoma differentiation associated factor 5; Retinoic acid-inducible gene-I

Core tip: The paramyxoviruses are a family of > 30 viruses that variously infect humans, other mammals and fish to cause diverse outcomes, ranging from asymptomatic to lethal disease, with the zoonotic paramyxoviruses Nipah and Hendra showing up to 70% case-fatality rate in humans. Here, we review the interferon antagonism strategies of paramyxoviruses, highlighting mechanistic differences observed between individual species and genera. We also discuss potential sources of this diversity, including biological differences in the host and/or tissue specificity of different paramyxoviruses, and potential effects of experimental approaches that have largely relied on *in vitro* systems.

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INTRODUCTION

Since the discovery more than 50 years ago of type I

interferons (IFNs) as the principal mediators of mammalian innate antiviral responses, it has become increasingly evident that infection by viruses depends on the capacity to counteract host cell IFN responses. Viruses have evolved diverse strategies to antagonise IFN responses, often by hijacking and modifying cellular regulatory pathways through the activity of specific viral IFN-antagonist proteins. Among the best-studied viruses in this respect are the paramyxoviruses, which include established human pathogens such as measles virus (MeV) and mumps virus (MuV), and emerging zoonotic viruses such as the henipaviruses Nipah virus (NiV) and Hendra virus (HeV). Although effective vaccines are available for MeV, it remains a leading cause of fatalities in children, with almost 140000 human deaths globally in 2010[1], while the henipaviruses show remarkable pathogenicity, with case-fatality rates between 40%-70% in humans[2-5].

The paramyxoviruses are a subfamily of the *Paramyxoviridae* family [order *Mononegavirales* (MNV)] of enveloped, non-segmented negative-strand RNA viruses (NNSV), which also includes the *Pneumovirus* subfamily[6,7]. Based largely on antigenic cross-reactivity and neuramidase activity paramyxoviruses are currently classified into seven genera[6,7]: *Rubulavirus*, *Avulavirus*, *Henipavirus*, *Morbillivirus*, *Respirovirus, Ferlavirus* and *Aquaparamyxovirus* (Table 1). Members of the paramyxovirus family show diverse tissue tropism and infect a variety of species in a fashion that does not appear to be specific to genus classification (Table 1). Because their relatively small genomes lack dedicated IFN-antagonist genes, paramyxoviruses generally encode IFN-antagonists as accessory protein isoforms encoded within their conserved P genes, another factor in genus classification[6]. These IFN antagonists broadly target several members of a select group of signalling molecules of the IFN system, including melanoma differentiation associated factor 5 (MDA5), retinoic acid-inducible gene- I (RIG)- I , IFN regulatory factor (IRF)-3, and signal transducers and activators of transcription (STAT)1 and STAT2, but use diverse mechanisms including proteosomal degradation, inhibition of phosphorylation, and subcellular mis-localisation. Intriguingly, the mechanisms can vary significantly between different genera and, in some cases, different species of the same genera.

Here we review the mechanistic data relating to paramyxovirus IFN antagonism with a focus on common and distinct features within the family, before discussing possible origins of the diversity within the family. Although much of the available research on paramyxovirus IFN antagonism has been restricted to *in vitro* studies, recent findings using *in vivo* infection and recombinant virus systems point to a pivotal role in pathogenicity that may provide potent targets for the development of new vaccines/antiviral therapeutics.

PARAMYXOVIRUS P GENE

While viruses with large genomes can encode dedicated IFN-antagonist proteins, the high error rates of the RNA-dependent RNA polymerase means that RNA viruses generally have restricted genome sizes, with the paramyxovirus genome containing only six principal genes to express essential structural/replication factors, specifically M (matrix), G/HN/H (attachment), F (fusion), L (polymerase), N/NP (nucleocapsid) and P (phosphoprotein) (Figure 1A). Thus the IFN-antagonists of RNA viruses are often encoded as "accessory" protein isoforms within one or more of the conserved genes[8,9]; in paramyxoviruses up to 9 proteins are encoded in the P gene, including V, C, and P proteins and a protein variously named W, D or I, which have established IFN antagonist functions.

Isoform expression from the *paramyxovirus P* gene is variously achieved by a conserved RNA-editing mechanism, and through the use of internal start codons and alternate open reading frames (ORFs). RNA editing is mediated by the viral RNA-dependent RNA polymerase

Table 1 Genus classification and major host species of the Paramyxovirinae subfamily

Genus	Virus	Major host
Morbillivirus	Measles virus[1]	Human
	Canine distemper virus	Canine
	Rinderpest virus	Bovine
	Peste-des-petits-ruminants virus	Caprine
	Phocine distemper virus	Phocine
	Cetacean morbillivirus	Cetacean
Rubulavirus	Mumps virus[1]	Human
	Parainfluenza virus 5 (previously, Simian virus 5)	Human
	Human parainfluenza virus 2, 4a and 4b	Human
	Mapuera virus	Chiropteran[2]
	Porcine rubulavirus	Porcine
Respirovirus	Sendai virus[1]	Murine
	Human parainfluenza virus 1, 3	Human
	Bovine parainfluenza virus 3	Bovine
Avulavirus	Newcastle disease virus[1]	Avian
	Avian paramyxoviruses 2-9	Avian
Henipavirus	Hendra virus[1]	Chiropteran/ equine /human[3]
	Nipah virus	Chiropteran/ porcine/ human[3]
	Cedar virus	Chiropteran[2]
Aquaparamyxovirus	Atlantic salmon paramyxovirus[1]	Piscine
Ferlavirus	Fer-de-Lance virus[1]	Serpentine
Unassigned	J-virus	Murine
	Beilong virus	Murine
	Tailam virus	Murine
	Menangle virus	Porcine
	Tioman virus	Chiropteran[2]
	Tupaia virus	Chiropteran[2]
	Salem virus	Chiropteran[2]
	Mossman virus	Chiropteran[2]
	Nariva virus	Chiropteran[2]
	Pigeon paramyxovirus 1	Avian

[1]Type species for each genus; [2]Virus isolated from chiropteran hosts, but pathology and broader host range is unknown; [3]Virus is highly pathogenic in humans, but humans are not a major host.

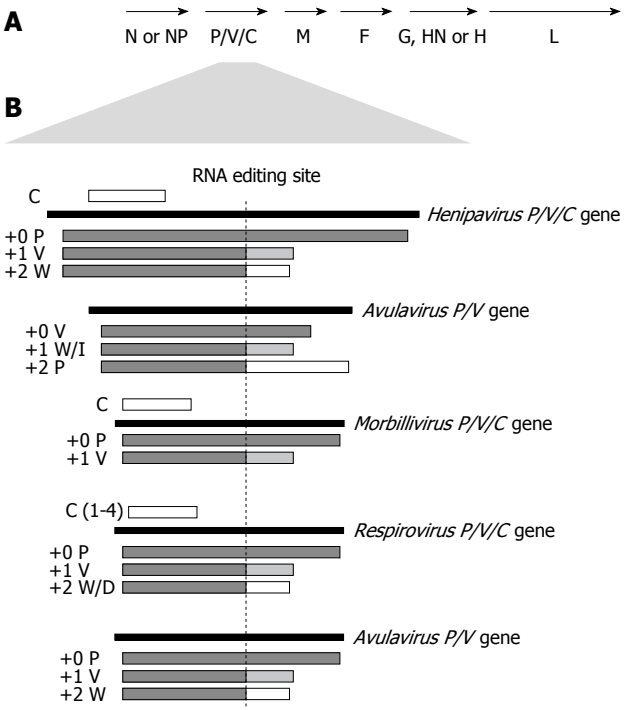

Figure 1 Coding strategies of *paramyxovirus P* genes. A: Genome organisation of the *Paramyxovirinae* subfamily; B: Paramyxoviruses express multiple proteins from the P gene through RNA editing to insert additional non-coded G nucleotides into P gene transcripts at the editing site (indicated), causing a frameshift in the downstream open reading frame (ORF) to generate distinct C-termini. Editing strategies of the 5 best-studied genera are shown, with proteins produced from unedited (+0), or edited (+1 or +2 frameshift) mRNA indicated below the P gene. Several members of the henipavirus, respirovirus and morbillivirus genera, but not the rubulaviruses or avulaviruses, produce one or more C proteins by translation from internal start codon(s) in alternate ORF(s) (indicated as a white bar above the P gene).

through the insertion of additional non-coded guanosine (G) nucleotides into P gene mRNA transcripts at a predetermined purine rich editing site. This causes a +1 or +2 frameshift in the downstream ORF[10-12] which results in the generation of two or three distinct proteins (P, V and W/D/I), which have common N-terminal sequences but unique C-termini (Figure 1B). A comparable editing process is used by Ebolavirus of the *Filovirus* family to produce isoforms from its G gene[13]. This mechanism is conserved among all paramyxoviruses examined except human parainfluenza virus (hPIV) 1 and the recently discovered cedar virus[14,15].

P protein, the polymerase cofactor essential to genome transcription/replication processes, is usually generated from the unedited ORF as the principal P gene product, with the production of edited RNA varying in a broadly genus-specific fashion (Figure 1B), although the +1 frameshift commonly encodes V protein and the +2 frameshift W/D/I[16,17]. Members of the *Rubulavirus* genus uniquely encode V protein in the unedited transcript, with P protein expression requiring editing (Figure 1B)[9], with c. 63% of the P gene mRNA transcribed unedited by the rubulavirus MuV, indicative of a particular requirement for high levels of V protein by these viruses[9]. The henipaviruses have the highest editing frequency of the paramyxoviruses: 66% to 94% of transcripts are edited, compared with c. 42% for MeV (*Morbillivirus* genus), and c. 31% for Sendai virus (SeV) (*Respirovirus* genus)[18-21]. Henipaviruses insert up to 11 additional G nucleotides[18], and in NiV-infected cells P transcripts are detected at the highest levels (c. 60%-100%) early in infection, with V and W transcripts peaking between 9.5-24 h post-infection (up to 59% and 37% respectively). This suggests that editing is regulated to enable particularly important roles for V and W late in infection[18], although other factors such as mRNA/protein stability are likely to affect the final levels of protein.

Henipaviruses, morbilliviruses, and respiroviruses use a start codon within the P gene in an alternate ORF to produce a C protein (Figure 1B), while the SeV P gene encodes up to five proteins other than P, V and W: four C proteins encoded by overlapping sequences in the +1 reading frame, and X protein, a truncated version of P protein translated from an internal start site[11,22,23]. HeV, but not NiV, encodes a putative SB (short basic) protein, homologous to SB of several viruses of other MNV families[21]. These differences in P gene coding capacity indicate different requirements of specific viruses for accessory proteins, possibly due to host/tissue specific aspects of IFN signalling (see below).

The V proteins are generally considered the principal IFN-antagonists of paramyxoviruses, and are the best studied of the P gene accessory proteins. However, there is increasing evidence that P, W, or C proteins of paramyxoviruses including NiV, MeV, and SeV play important roles in IFN antagonism by distinct mechanisms. Thus, it seems that most if not all P gene accessory proteins have evolved for roles in immune evasion as important pathogenicity factors[24-28]. Consistent with important roles in infection, V proteins show high conservation in the unique C-terminal region (Figure 2)[29-31], including absolute conservation of seven conserved cysteine residues and a histidine, which form a zinc-finger domain (highlighted in Figure 2). In the parainfluenza virus 5 (PIV5) V protein (*Rubulavirus* genus), two zinc atoms are coordinated by two loops, incorporating V residues H171, C190, C215, C218, and C194, C206, C208, C211 respectively[31,32], and mutations of these residues disrupt certain IFN inhibitory functions (see below), although the role of zinc-binding is not known. The C-terminal region is also important to the formation of oligomeric structures of V proteins and certain innate immune factors important to IFN antagonism[33].

TYPE I IFN SYSTEM

Mammalian cell responses to infection depend on the detection of pathogen-associated molecular patterns (PAMPs) produced during microbial infection and replication, such as single-stranded RNA (ssRNA), double-stranded RNA (dsRNA) and RNA with exposed/uncapped 5' triphosphates that are generated by RNA viruses[34,35]. Detection of virus components is principally

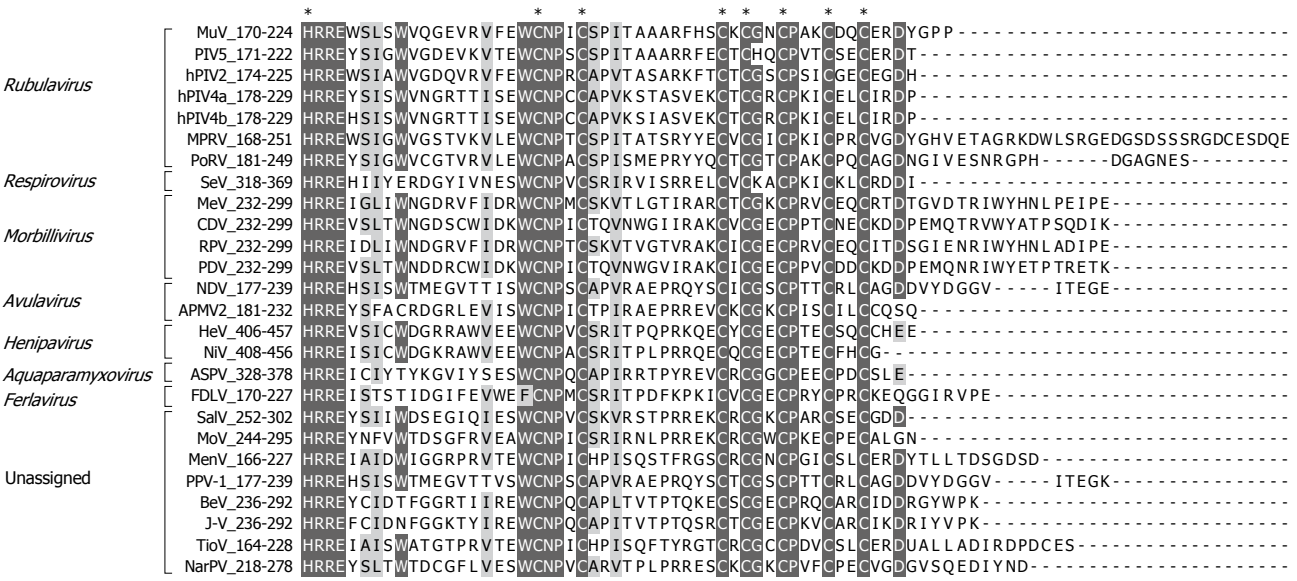

Figure 2 Conserved residues in the paramyxovirus V C-terminal domain. Paramyxovirus V protein C-terminal sequences are aligned with identical and similar residues highlighted. Asterisks indicate absolutely conserved histidine and cysteine residues involved in zinc-binding (see text for details). Residue numbers are indicated in the sequence titles. MuV: Mumps virus; PIV5: Parainfluenza virus 5; hPIV: Human PIV; MPRV: Mapuera virus; PoRV: Porcine rubulavirus; SeV: Sendai virus; MeV: Measles virus; CDV: Canine distemper virus; RPV: Rinderpest virus; PDV: Phocine distemper virus; NDV: Newcastle disease virus; APMV2: Avian paramyxovirus 2; HeV: Hendra virus; NiV: Nipah virus; ASPV: Atlantic Salmon Paramyxovirus; FDLV: Fer-de-Lance virus; SalV: Salem virus; MoV: Mossman virus; MenV: Menangle virus; PPV-1: Pigeon paramyxovirus 1; BeV: Beilong virus; J-V: J-virus; TioV: Tioman virus; NarPV: Nariva virus.

mediated by three types of PAMP-recognition-receptors (PRRs): Toll-like receptors (TLRs) and RIG- I -like receptors (RLRs), thought to be the main receptors responsible for type I IFN (IFNα/β) induction, and nucleotide-oligomerisation domain-like receptors[36].

TLRs are trans-membrane proteins expressed at the plasma membrane or on intracellular structures such as endosomes and the endoplasmic reticulum[37,38] to detect extracellular viral nucleic acids such as dsRNA (TLR3)[37-40] and G/U-rich ssRNAs (TLR7)[38]. By contrast, the almost ubiquitously expressed RLR helicases RIG- I and MDA5 detect viral dsRNA in the cytoplasm of infected cells[36,41-47]; RIG- I also recognises cytoplasmic 5' tri-phosphorylated and uncapped viral ssRNA[48-50]. RNA-activated MDA5 and RIG- I interact with the mitochondrial membrane-associated adaptor protein IFNβ promoter stimulator 1 (IPS-1, also known as MAVS, VISA, or CARDIF) *via* their caspase activation and recruitment domains (CARDs) to trigger downstream signalling (Figure 3). TLRs activate distinct pathways (Figure 3), but RLR and TLR signalling converges with the phosphorylation of the constitutively expressed cytoplasmic transcription factors IRF-3, as well as nuclear factor κB (NF-κB), causing their translocation into the nucleus to activate the transcription of early type I IFNs (IFNβ and IFNα4)[36,51-56]. Most human cell types can produce type I IFNs in response to infection, with "professional" IFN-producing immune cells including plasmacytoid DCs and macrophages being major producers during infection, due to constitutive expression of IRF-7 (which requires induction in other cell types) and the use of alternative TLR-9 pathways[57]. Importantly, paramyxoviruses can induce type I IFN expression through RIG- I , MDA5 and TLR pathways (Figure 3)[42,58,59].

Type I IFNs signal in autocrine and paracrine fashion, binding to the ubiquitously expressed IFNα/β receptor (IFNAR) to activate the Janus kinase (JAK)/STAT signalling pathway (Figure 4), resulting in the phosphorylation and nuclear translocation of STAT1 and STAT2 proteins. In the form of a heterotrimeric complex [IFN-stimulated gene factor 3 (ISGF3)] which incorporates IRF-9, STAT1 and STAT2 trans-activate hundreds of IFN-stimulated genes, many of which encode known antiviral proteins including protein kinase R, which inhibits translation of mRNAs[60]; 2'5'-oligoadenylate synthetase, which activates RNase L to effect degradation of ssRNA[60]; Mx GTPase proteins that interfere with the growth of certain viruses including the paramyxoviruses[52]; and PML, which has antiviral properties but with unresolved mechanisms[52]. IRF-7 is also up regulated to activate a positive feedback loop by forming heterodimers with IRF-3 (Figure 3) inducing "late" IFNα subtypes for prolonged responses to infection[61].

Although signalling through STAT1/2 heterodimers is essential to type I IFN responses, type I IFN activates other complexes including homodimers of STAT1 and STAT3 and STAT1-STAT3 heterodimers, which have different gene specificity or regulatory roles[62], and recent data suggest that STAT2 can also effect STAT1-independent antiviral functions[63]. Thus, type I IFN activation can affect diverse gene expression through distinct pathways. STATs are also critical to signalling by type Ⅱ and Ⅲ IFNs and various other cytokines[54,64] such as interleukin (IL)-6[65,66], presenting potential targets for viral inhibition of several immune signalling systems.

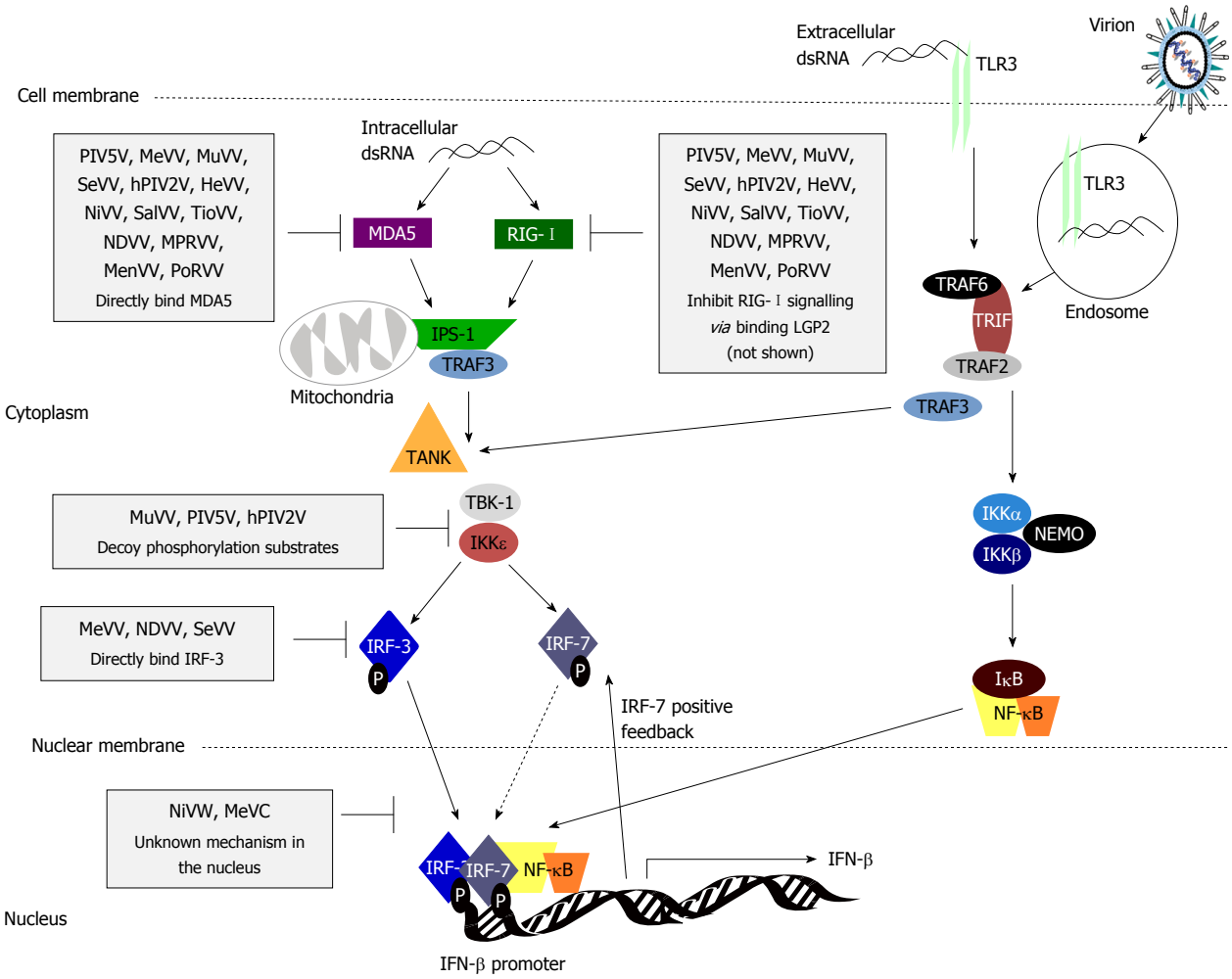

Figure 3 Type I interferon induction is inhibited by paramyxovirus interferon-antagonist proteins at multiple stages. Pathogen-associated molecular patterns (PAMPs) generated during virus infection, such as dsRNA, are recognised by PRRs including endosomal/surface expressed Toll-like receptor 3 (TLR3) and cytoplasmic retinoic acid-inducible gene- I (RIG- I)/melanoma differentiation associated protein 5 (MDA5). TLR3 signals through the adaptor molecule Toll/interleukin-1 receptor domain-containing adaptor inducing interferon (IFN)β (TRIF), which recruits tumor necrosis factor receptor-associated factor (TRAF)2 to activate the inhibitor of nuclear factor κB (NF-κB) kinase (IKK)α/β kinases to phosphorylate inhibitory inhibitor of NF-κB (IκB), triggering its degradation and activation/nuclear translocation of NF-κB. TLR3 signalling *via* TRAF3 results in phosphorylation/activation of IFN regulatory factor (IRF)-3, causing its homodimerisation, or heterodimerisation with IRF-7 in professional IFN producing/IFN-primed cells, and translocation into the nucleus where, with NF-κB and activating transcription factor 2 (ATF2)/c-jun (not shown), it activates early type I IFN transcription. RIG- I and MDA5 also induce phosphorylation of IRF-3 following recognition of cytoplasmic PAMPs in infected cells *via* interaction with the mitochondrial membrane protein IFNβ promoter stimulator 1 (IPS-1), which recruits and activates TANK and the TANK-binding kinases (TBKs). TBK-1 and IKKε *via* the E3 ubiquitin ligase TRAF3. Many paramyxoviruses target this pathway; steps commonly targeted are indicated (black bars) with specific examples of the paramyxovirus proteins responsible (see text for details). DC: Dendritic cell; PIV5: Parainfluenza virus 5; MeV: Measles virus; MuV: Mumps virus; SeV: Sendai virus; hPIV: Human PIV; HeV: Hendra virus; NiV: Nipah virus; SalV: Salem virus; TioV: Tioman virus; NDV: Newcastle disease virus; MPRV: Mapuera virus; MenV: Menangle virus; PoRV: Porcine rubulavirus; LGP2: Laboratory of genetics and physiology 2.

CELLULAR TARGETS OF PARAMYXOVIRUS IFN ANTAGONISTS

A large body of evidence indicates that viruses/IFN-antagonist proteins generally target multiple steps in the IFN system[52,67,68]. The requirement for this broad targeting probably relates to factors such as differences in the kinetics of viral IFN-antagonist expression compared with the mounting of IFN responses, the contribution of infected cells and non-infected professional IFN producing cells, and the overall antiviral potency of the IFN system. Most paramyxoviruses can inhibit both IFN induction and signalling by targeting several cellular proteins. Intriguingly, although paramyxoviruses generally target common factors including MDA5, IRF-3 and STATs, the mechanisms of inhibition show significant divergence between different viruses.

Targeting of MDA5

The V proteins of at least 13 paramyxoviruses tested bind to MDA5 to inhibit IFN induction[32,69-71]. Rinderpest virus (RPV) may differ, as it appears to use the C protein rather than V to inhibit MDA5 signalling, although the binding of RPV V to MDA5 has not been examined[72]. The V proteins of PIV5, hPIV2, MuV, MeV, NiV, HeV, SeV, Mapuera virus (MPRV), Menangle virus (MenV)

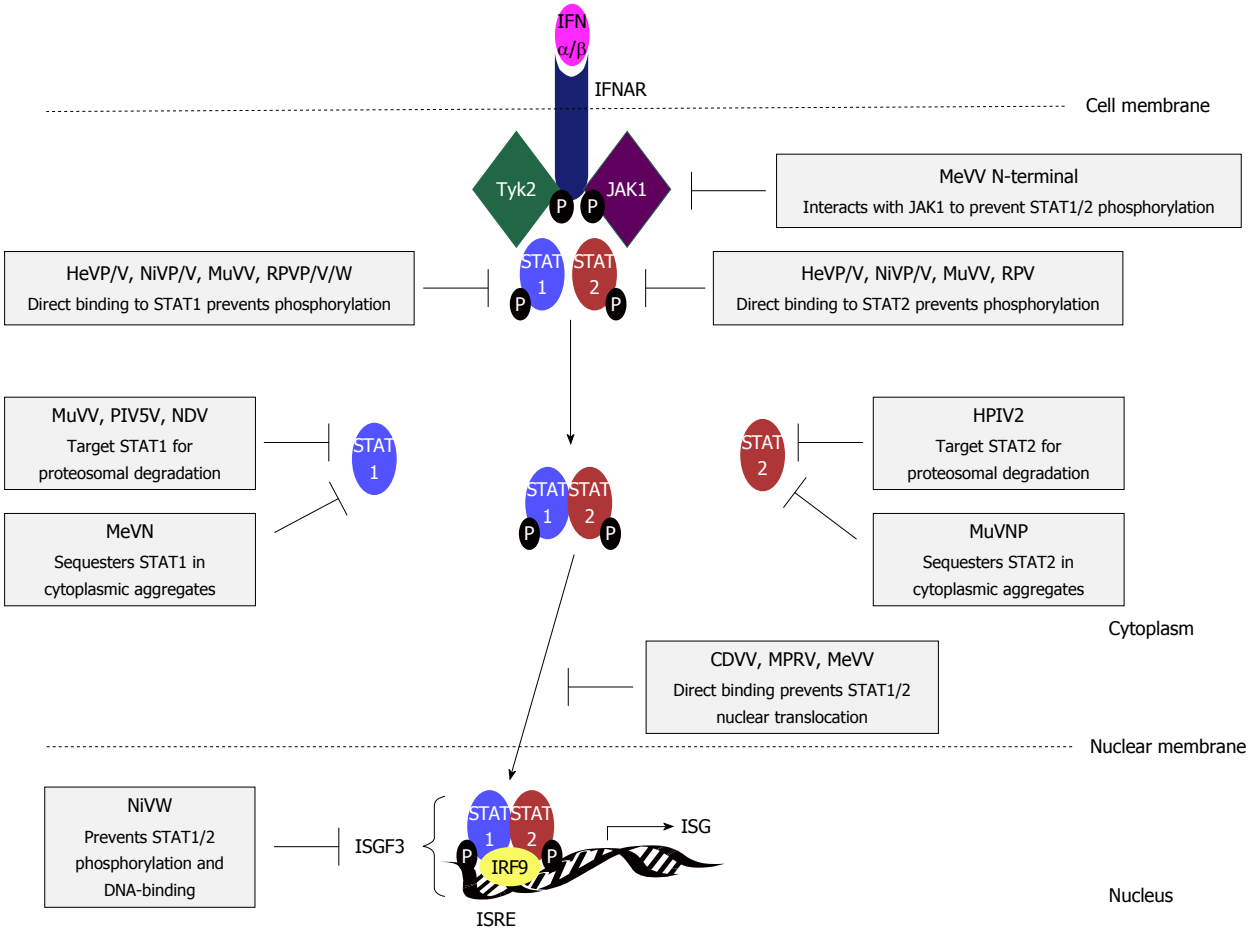

Figure 4 Interferon signalling pathways are targeted by paramyxovirus interferon-antagonist proteins through diverse mechanisms. Interferon (IFN)β binds to type I IFN receptor subunits IFNα/β receptor (IFNAR)1 and IFNAR2, causing dimerization, activation and phosphorylation of the receptor-associated kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2), to create docking sites for the SH2 domains of signal transducers and activators of transcription (STAT)1 and 2. STAT1 and 2 are phosphorylated by Tyk2 and JAK1 respectively, and form a heterodimer that translocates into the nucleus, forming the heterotrimeric transcription factor complex IFN-stimulated gene factor 3 (ISGF3) with IFN regulatory factor (IRF)-9. ISGF3 binding to IFN stimulatory response element (ISRE) sequences in the promoters of hundreds of IFN-stimulated genes (ISGs) activates the transcription of antiviral and immune-modulatory proteins to establish an antiviral state in infected and neighbouring cells, and contribute to shaping the adaptive immune response. STAT1 and/or STAT2 are targeted by almost all paramyxoviruses through the activity of several IFN antagonists by mechanisms that are reported to differ significantly; mechanisms and specific viral proteins responsible are indicated (see text for details). HeV: Hendra virus; NiV: Nipah virus; MuV: Mumps virus; RPV: Rinderpest virus; MeV: Measles virus; PIV5: Parainfluenza virus 5; NDV: Newcastle disease virus; hPIV: Human PIV; CDV: Canine distemper virus; MPRV: Mapuera virus.

and Salem virus (SalV) were shown to bind a specific region within/proximal to residues 701-816 of the MDA5 helicase domain, independently of the MDA5 ligand dsRNA[70,71,73], thereby blocking dsRNA-MDA5 interaction[70]. Although a recent study of PIV5 V identified a change in the dsRNA-binding properties of MDA5 when V was co-expressed, rather than a complete inhibition, suggesting that V may still allow non-cooperative dsRNA binding[74]. In addition, the V proteins of PIV5, MenV, and SalV might have further specialist antagonistic functions, as yeast two-hybrid assays indicated that they interacted with multiple distinct regions of MDA5[70]. A crystal structure of PIV5 V in complex with MDA5 has demonstrated that V unfolds the ATPase domain of MDA5, which allows it to bind a region normally hidden beneath the helicase fold[74]. This unfolding disturbs the ATPase hydrolysis site, and it was shown using MeV V that increasing concentrations of V correlate with decreasing ATPase activity[74].

The MDA5 binding site has been mapped to the C-terminal region of the V proteins of PIV5, MeV, MuV, Newcastle disease virus (NDV), NiV, HeV and SeV[32,69-71,75,76], with conserved residues of the zinc-finger critical to the interaction. However, the precise molecular details differ between specific paramyxoviruses, with conserved cysteine residues in the large zinc finger loop, but not the smaller loop, of PIV5 V and NiV V dispensable for antagonism of IFN induction[32], whereas MuV V and MeV V required all conserved cysteine residues[32]. A crystal structure of MDA5:PIV5 has shown PIV5 V to have six residues (174, 175, 177, 179, 184 and 197) involved in forming the interface with MDA5, only some of which are conserved with other paramyxovirus V proteins[74].

Targeting of RIG-I via laboratory of genetics and physiology 2 protein

In contrast to MDA5, V proteins do not bind directly to RIG-I, nor inhibit RIG-I oligomersation or dsRNA-

binding[70], which has been assumed to indicate that they have no direct role in inhibiting RIG- I activation, but rather target downstream signalling components such as IRF-3 (see below). However, recent data has indicated that V proteins can inhibit RIG- I by interaction with another cellular helicase, the laboratory of genetics and physiology 2 (LGP2)[73], *via* a region of LGP2 homologous to the V protein binding region in MDA5[71,73]. The interaction appears to be dependent on the unique C-terminal domain of V protein, as PIV5 P protein did not bind to LGP2, but the C-terminal domains of MeV and MuV V proteins were necessary and sufficient for the interaction[71,73]. Importantly, V proteins were able to inhibit RIG- I signalling only in cells where LGP2 was coexpressed[73], and RIG- I - LGP2 interaction was detected only in cells expressing V protein, suggesting that V facilitates or mediates this interaction to shutdown RIG- I activation[73]. Because LGP2 is homologous to RIG- I and MDA5, but lacks the CARD domain to activate downstream signalling, it is thought to be a negative regulator of IFN induction, consistent with the inhibitory effects of V protein expression. However, there is evidence that LGP2 can positively regulate IFN induction under some conditions[77-80], so the precise mechanisms of V protein/LGP2 antagonism of RIG- I remain to be determined.

Inhibition of IRF-3 activation

In addition to inhibition of PRRs, paramyxoviruses target downstream signalling components to prevent activation of IRF-3, potentially as a mechanism to inhibit signalling by both RLRs and TLRs (Figure 3). Rubulaviruses including MuV, hPIV2, and PIV5 use V protein as a decoy substrate for the IRF-3 kinases TANK-binding kinase 1 (TBK-1) and inhibitor of NF-κB kinase (IKK)ε (Figure 3), both inhibiting phosphorylation of IRF-3 and facilitating IKKε/TBK-1 polyubiquitination and degradation to prevent further signalling[81].

Henipavirus V proteins do not cause IKKε/TBK-1 degradation[81] or block TLR-3/IRF-3 dependent signalling[76,81]. For henipaviruses, this appears to be a function of the W protein, as NiV W, although having no effect on MDA5 signalling, inhibited TLR-3-dependent phosphorylation of IRF-3[82]. It is possible this is due to binding and sequestration of inactive IRF-3 in the nucleus where NiV W localises, to prevent interaction with cytoplasmic IKKε/TBK-1[82]. This model is consistent with the reported importance of NiV W protein nuclear localisation to its inhibition of TLR3-dependent IFN induction[82]. MeV C protein also inhibits IFN induction, correlating with its nuclear localisation[83], although MeV C does not affect IRF-3 directly, and appears to have an undetermined nuclear target[83]. By contrast, cytoplasmic NDV and SeV V protein bind directly to IRF-3, thereby preventing its nuclear translocation[76]. Thus, paramyxovirus targeting of IRF-3-mediated signalling involves mechanisms that appear to differ significantly between species.

Targeting of STATs by rubulaviruses: degradation and mis-localisation

Almost all rubulavirus V proteins target STAT1 or STAT2 for degradation by the host-cell proteosomal pathways[84-87] through assembly of a V-degradation complex (VDC) containing V protein, STAT1, STAT2, and components of an E3 ubiquitin ligase complex, specifically the UV damage-specific DNA binding protein 1 (DDB1), and Cul4A[88-92], which likely mediate the STAT1/2 polyubiquitination[93]. *In vitro* studies/crystallographic analysis of the PIV5 V-DBB1 complex have indicated that both the N-terminal and unique C-terminal regions of PIV5 V are required for VDC assembly and STAT1 degradation[33,88,93,94]. Intriguingly, although some rubulaviruses target only STAT1 or STAT2 for degradation (see Figure 4 for details)[95,96], both STATs are required, with the non-degraded STAT acting as a "co-factor"[97,98].

The MuV V protein VDC polyubiquitinates and degrades not only STAT1, but also STAT3[84,99], such that MuV V protein can inhibit STAT3-dependent transcriptional activation by IL-6 and v-Src[99]. MuV targeting of STAT3 is independent of STAT1 targeting, as a point mutation abrogating targeting of STAT3 did not affect STAT1[100], and STAT3 degradation does not require the STAT2 "cofactor"[99]. STAT3 targeting by the V protein of MuV is also highly specific to this species, as the V proteins of the rubulaviruses MPRV, hPIV2 and hPIV4 do not reduce cellular levels of STAT3[87,101,102].

Intriguingly, the V proteins of hPIV4a and hPIV4b do not degrade STATs or measurably affect their localisation or phosphorylation, but still bind to STAT1, STAT2 and other VDC components[101]. While these viruses appear to lack the ability to antagonise STAT signalling, the specific binding capacity of the proteins is suggestive of a previous role in STAT antagonism, which may have been lost due to changes in selective pressures[101].

MPRV V protein, by contrast with those of other rubulaviruses, binds to STAT1 and STAT2 to prevent their nuclear translocation without inducing degradation[102]. This is similar to reports for the V proteins of the henipaviruses and morbilliviruses (see below), except in that MPRV V does not inhibit STAT1 phosphorylation and can bind to STAT1 and STAT2 independently[102]. A similar mechanism may be used by the MuV NP protein, which co-localises with STAT2 in punctate aggregates in the cytoplasm of infected cells[99], indicating that NP protein, like P protein, can mediate both replication and IFN antagonist functions.

Targeting of STATs by avulaviruses

In common with rubulaviruses such as PIV5, the avulavirus NDV targets STAT1, but not STAT2, for degradation. Deletion of the C-terminal region of V protein, or deletion of both V and W C-termini by disruption of the RNA editing site, prevented STAT1 degradation by recombinant NDV[95]. As little difference was observed between virus deleted for both V and W, and virus deleted

for the V protein C-terminal domain alone, V protein appears to be the major player, and consistent with this, NDV V but not NDV W degraded STAT1 in transfected cells[95].

Targeting of STATs by morbilliviruses

MeV V binds STAT1 and STAT2 through distinct sites in its N-terminal and C-terminal regions[103], respectively, indicating that targeting of STAT2 independently of STAT1 is important to this virus. MeV V protein does not degrade STATs[104], but has been reported by different laboratories to use several distinct mechanisms, including inhibition of STAT nuclear translocation without affecting STAT phosphorylation[103-105], and inhibition of STAT1 and STAT2 phosphorylation due to interaction of its N-terminal domain with JAK1[106,107]. Canine distemper virus (CDV) and RPV V proteins also inhibit IFN-activated STAT1/STAT2 nuclear import[108,109], with RPV V protein, but not that of CDV, inhibiting STAT1/2 phosphorylation.

MeV V also interacts with IRF-9, which is likely to affect ISGF3 formation (Figure 4)[104], and with STAT3[104], a property thus far restricted in the paramyxovirus family to MeV and MuV V proteins[99,100,104,110]. HeV V and PIV5 V have been shown to lack STAT3 binding function, and while SeV infection can inhibit IFNα-dependent STAT3 phosphorylation, this appears to relate to upstream effects on Tyk2 rather than STAT3 directly[111]. STAT3 binding by other paramyxovirus V proteins, however, has not been investigated.

MeV N protein also inhibits STAT1/2 signalling[112], indicating a particular importance of STAT inhibition to MeV, and co-localises with STAT1 in cytoplasmic aggregates in infected cells, analogously to MuV NP[99,104]. STAT2 also co-localised with MeV N in aggregates, but with reduced frequency compared with STAT1[104].

STAT targeting by respiroviruses: the importance of C proteins

STAT targeting by respiroviruses differs significantly from other paramyxoviruses, due to the expression of additional proteins from the P gene (Figure 2), including four C proteins by hPIV1[14,113], which does not express V or W. The C' protein of hPIV1 binds and sequesters STAT1 in perinuclear aggregates, suggesting that the C proteins may be sufficient for IFN antagonism by this virus[114]. SeV C proteins (C', C, Y1 and Y2), also bind to STAT1 and prevent signalling and, importantly, the functions of the individual C proteins appear non-redundant, as knockout of all four proteins is required to completely prevent IFN antagonism in infected cells[115,116]. Data regarding the mechanisms of SeV C proteins activity are conflicting[116-121], with some reports suggesting that C and C', but not Y1 or Y2, cause STAT1 mono-ubiquitination/degradation[116,117] dependent on the C protein N-termini[118,119], while others reported no reduction in STAT1 expression but indicated inhibition of STAT1 and STAT2 phosphorylation by the C proteins, independently of their N-termini[120,121].

STAT targeting by henipaviruses: the roles of P, V and W

The henipavirus P, V and W proteins can bind to STAT1 and STAT2 through the shared N-terminal region[122,123] to prevent STAT1/2 phosphorylation and activation by holding them in high molecular weight complexes[110,123-125]. Transfection studies indicate that P, V and W have differing capacities to inhibit STAT signalling, with P protein the least effective[125]. This is consistent with the hypothesis that the V and W accessory proteins have evolved to enable specific, distinct roles as IFN-antagonists, sequestering STATs in the cytoplasm and the nucleus, respectively[82,122,125], whereas P protein functions principally as the polymerase cofactor, but can arrest STATs in the cytoplasm. Mutation of the shared G121 residue was found to specifically ablate STAT1 binding by V, W and P, without affecting P protein polymerase cofactor function, enabling the production of recombinant NiV impaired for STAT antagonistic functions to confirm that inhibition of STAT1 phosphorylation in NiV infected cells is due to P/V/W binding[122,126]. In wild-type NiV-infected cells, but not those infected with the mutant NiV, unphosphorylated STAT1 localised exclusively to the nucleus, similar to cells expressing W protein alone, suggesting NiV W has the predominant role in blocking STAT signalling in infected cells[122].

DIFFERENT MECHANISMS OF IMMUNE EVASION: EVOLUTION OR EXPERIMENT?

Although there is abundant evidence that *paramyxovirus P* gene-encoded proteins can antagonise IFN responses by diverse species-specific/genera-specific mechanisms, the source of this diversity is currently unclear. A major caveat of the available data is its heavy reliance on *in vitro* studies, particularly transfection studies of single IFN-antagonist proteins. Although these approaches enable highly specific analyses of the properties of particular IFN-antagonists, including mapping/mutagenesis studies, the potential to generate artefactual data due to the absence of other viral factors and/or non-physiological expression levels is a significant concern. Indeed, several transfection studies in different laboratories have generated conflicting mechanistic data for the same viral protein, including SeV C and MeV V protein[103-107,116,117,120,121], suggesting that some reported differences between IFN antagonists of different paramyxovirus species/genera might arise from experimental rather than biological differences. Importantly, however, recent studies comparing in parallel the functions of V proteins from panels of paramyxoviruses have confirmed clear divergence in specific mechanisms/interactions[70,71,76], indicating genuine divergence at the molecular level.

Recent studies have also directly compared IFN-antagonist protein expression/functions in transfected and

infected cells, identifying clear differences. Notably, one study reported that while henipavirus V and W proteins profoundly inhibit IFN/STAT signalling in transfected cells, no inhibition was apparent in infected cells, which appeared to relate to the higher expression of V/W proteins in transfected cells[127]. This suggested that STAT inhibition by V and W does not have significant roles in infected cells, but it seems unlikely that viruses would evolve proteins that can specifically target factors of the IFN response and impede their function by sophisticated mechanisms were this not important at some stage of infection. While *in vitro* infection approaches are clearly closer to natural infection than transfection, they also use controlled *in vitro* conditions including the inoculation of cultured monolayers of specific cell types with precise multiplicities of infection, and treatments with specific concentrations of IFNs. By contrast, in natural infection the kinetics of viral protein expression and induction of the IFN system is highly dynamic, involving both infected cells and professional IFN-producing cells, and factors such as the infectious dose, route of infection, host species, and infectious spread to specific tissues can vary greatly, significantly affecting requirements for IFN antagonism and the disease outcome[128]. Thus, the diverse mechanisms of IFN antagonism identified in transfection studies may have vital roles in infection *in vivo*.

Importantly, IFN antagonism has been implicated as a key factor in host and tissue specificity, with PIV5 showing limited host range dependent on the capacity of the V protein to bind to STAT2 from different species[129-132], whereas NiV V blocks IFN signalling in cells of many species, consistent with its broad infectious range[82,110,124,126]. Tissue-specific antagonism of IFN has also been reported for NiV, which induces an IFN response in endothelial but not neuronal cells, correlating with differential subcellular localisation of NiV W[133].

A genuine appreciation of the importance of specific IFN-antagonistic mechanisms to pathogenicity, however, requires the use of recombinant virus systems and *in vivo* pathogenicity models. Recent advances in this area include reports that recombinant hPIV2 impaired for V protein antagonism of MDA5 is attenuated in rhesus monkeys[134-136], and that the severity of clinical signs in MeV-infected monkeys was reduced by mutation of the P/V proteins to prevent inhibition of STAT1[137]. In addition, the deletion of V or C proteins from MeV caused attenuation in mice, but V deletion alone resulted in restricted spread in the brain[138], supporting the hypothesis that specific mechanisms of IFN-antagonism are important to infection of certain tissues. Deletion of the V C-terminal domain in recombinant NiV also reduced pathogenicity in a hamster model[123-125,139], possibly due to IFN-antagonist functions of the V C-terminal domain, such as the targeting of MDA5.

Of paramount importance to delineating the roles of specific mechanisms of IFN antagonism in pathogenicity will be the extension of *in vivo* studies to include genetically modified animals deficient in specific IFN signalling processes. For example, recent research indicated that SeV pathogenicity is increased in MDA5 knockout mice[140], suggesting that this might provide a useful model to investigate the importance of MDA5 antagonism in *in vivo* infection.

CONCLUSION

A substantial body of data from the past c. 15 years has provided key insights into the immune evasion strategies of paramyxovirus IFN-antagonists, indicating that they employ a remarkable array of mechanisms to target essential factors of the IFN response, with the limited *in vivo* infection data indicating that these functions are essential to pathogenicity. However, as much of the current mechanistic data comes from *in vitro* transfection approaches, their importance to natural infection remains largely unresolved. Future studies employing *in vivo* infection models, recombinant virus systems and genetically modified animals should begin to unravel in detail the interactions of paramyxoviruses with the IFN system *in vivo*. This is likely to result in the identification of new potential targets for the development of vaccines and antivirals required for the treatment of established prolific human pathogens such as MeV, as well as emerging zoonotic threats including NiV and HeV.

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TOPIC HIGHLIGHT

Gualtiero Alvisi, PhD, Assistant Professor, *Series Editor*

Viral proteins and Src family kinases: Mechanisms of pathogenicity from a "liaison dangereuse"

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Abstract

To complete their life cycle and spread, viruses interfere with and gain control of diverse cellular processes, this most often occurring through interaction between viral proteins (VPs) and resident protein partners. Among the latter, Src family kinases (SFKs), a class of non-receptor tyrosine kinases that contributes to the conversion of extracellular signals into intracellular signaling cascades and is involved in virtually all cellular processes, have recently emerged as critical mediators between the cell's infrastructure and the viral demands. In this scenario, structural or *ex novo* synthesized VPs are able to bind to the different domains of these enzymes through specific short linear motifs present along their sequences. Proline-rich motifs displaying the conserved minimal consensus PxxP and recognizing the SFK Src homology (SH)3 domain constitute a cardinal signature for the formation of multiprotein complexes and this interaction may promote phosphorylation of VPs by SFKs, thus creating phosphotyrosine motifs that become a docking site for the SH2 domains of SFKs or other SH2 domain-bearing signaling molecules. Importantly, the formation of these assemblies also results in a change in the activity and/or location of SFKs, and these events are critical in perturbing key signaling pathways so that viruses can utilize the cell's machinery to their own benefit. In the light of these observations, although VPs as such, especially those with enzyme activity, are still regarded as valuable targets for therapeutic strategies, multiprotein complexes composed of viral and host cell proteins are increasingly becoming objects of investigation with a view to deeply characterize the structural aspects that favor their formation and to develop new compounds able to contrast viral diseases in an alternative manner.

Key words: Interaction; Phosphotyrosine; Proline-rich motif; Src homology 2 domain; Src homology 3 domain

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INTRODUCTION

Although not strictly meeting the definition of living organisms, viruses can replicate and spread, provided that they infect host cells to produce a new offspring. In this regard, viruses are obligate intracellular parasites that have evolved complex strategies to complete their life cycle, consisting of contrasting and even evading innate defense mechanisms of the host cell for which they have a tropism so as to take control over key cell pathways[1,2]. Therefore, they interfere with diverse cellular processes, especially those involving replication of genetic material, protein translation and trafficking, with viral structural and non-structural proteins undergoing post-translational modifications, such as phosphorylation, ubiquitination, glycosylation and cleavage prior to or after interacting with a vast part of

the host's proteome, thereby forming new multiprotein assemblies[2]. Indeed, much effort has been made over the last few decades to dissect the cellular signaling pathways hijacked by viruses and the underlying molecular mechanisms, leading to the identification of several host cell factors playing a major role in the viral life cycle. Among these, Src family kinases (SFKs), a class of non-receptor tyrosine kinases comprising of eight members (Src, Yes, Fyn, Fgr, Lyn, Hck, Lck and Blk), have emerged as critical mediators between the cell infrastructure and the viral demands. Although traditionally known as molecular switches situated right beneath the plasma membrane, from there relaying extracellular cues and governing signal transduction, SFKs localize to virtually all the other cell compartments, thereby regulating a wide spectrum of cellular processes, such as growth, viability, cell cycle and metabolism[3,4]. To gain a deeper understanding of how SFKs exert their function and how viruses can benefit from the interplay with SFKs, it is necessary to bear in mind the complexity of the multidomain organization and the mechanisms of activation of SFKs themselves. From the N- to the C-terminus, their structure consists of: (1) the Src homology (SH) 4 domain, a unique region that becomes myristoylated and/or palmitoylated for membrane association of SFKs; (2) the SH3 domain, which binds specific proline-rich motifs (PRMs); (3) the SH2 domain, which recognizes phosphotyrosine motifs; (4) the SH2-kinase linker; (5) a catalytic SH1 domain; and followed by (6) a C-terminal tail implicated in the downregulation of SFKs (Figure 1). The activity of SFKs is mainly modulated by the phosphorylation state of 2 critical tyrosine residues, Tyr416 and Tyr527 (based on the amino acid numbering of chicken c-Src and corresponding to Tyr419 and Tyr530 in humans), with opposing effects: the former, which lies in the activation loop, is subjected to autophosphorylation when the SFK is activated, whereas the latter is targeted by C-Src tyrosine kinase[5], resulting in the inactivation of the tyrosine kinase. The latter event is induced by a closed conformation of the SFK through 2 major intramolecular inhibitory interactions, binding of the C-terminal phosphotyrosine (Tyr527) itself to the SH2 domain and interaction of a polyproline type Ⅱ helical motif (PPⅡ) in the SH2-kinase linker with the SH3 domain. On the other hand, multiple events can induce disruption of such inhibitory mechanisms, such as dephosphorylation of the tail, with its displacement from the SH2 domain and/or displacement of the PPⅡ motif from the SH3 domain, ultimately resulting in the full activation of SFKs[6]. All these features, either functional or structural, can be exploited one at a time or in combination by most, if not all, viruses to take over the cell machinery, from the cell entry, all through the genome replication until the release of new particles. As to internalization, viral particles induce, upon interaction of cognate membrane receptors, activation of SFKs, which, as apical cellular transducers in receptor-mediated cellular signaling, take part in the activation of clathrin-, caveolin-dependent endocytic pathways, or the alternate mechanism based upon macropinocytosis and are subsequently taken up by host cells for uncoating and genome replication[7-10]. Another different mode of viral uptake into the host cell occurs in polarized cells and is mediated by SFKs, which are activated upon virus attachment to the plasma membrane, so that tight junction barrier function is perturbed to allow viruses to reach their specific receptors at the baso-lateral side of epithelial cells. As a result, viral particles can be endocytosed[9] or participate in relocalizing viral receptors from the baso-lateral to the apical membrane surface in response to cytokines released by infected macrophages with subsequent entry into the epithelial cells[10].

Thus, regardless of the viral species, it can be stated that SFKs are involved in the cell entry of viral particles simply by "doing their duty" as a component of signalosomes, that is, by relaying the extracellular cues, in this case consisting in viral ligands that bind to cell receptors, to downstream effector molecules and preparing the cellular environment to take up virions by the different mechanisms of endocytosis. An actual interaction between SFKs and viral proteins (VPs) does not occur in this early phase of infection, instead taking place only after uncoating and *ex novo* synthesis of viral gene products. This association can be mediated by the non-catalytic (SH3 and SH2) and catalytic (SH1) domains of SFKs, thereby resulting in directing the localization or affecting the activity of SFKs themselves, in order to best support genome replication, particle assembly and spread.

This review offers a brief summary of the current knowledge of the molecular mechanisms underlying the interactions between SFKs and VPs, as well as the consequences thereof, so as to highlight common or rather specific structural motifs that might become molecular targets for disrupting such associations and to provide a new perspective in managing viral infections.

INTRACELLULAR VIRAL HIJACKING OF SFKS

It has been largely described that a plethora of viruses, once they enter the cell, utilize SFKs to foster the different steps of the viral life cycle, although the molecular mechanisms have not been elucidated in many cases. However, accumulating evidence indicates that direct interaction of VPs with SFKs results in the activation of SFKs with subsequent (1) phosphorylation of VPs, which acquire new functional properties (Figure 2A-C), or (2) delocalization of SFKs to cell compartments targeted by specific VPs, where SFKs can exert their catalytic and non-catalytic action (Figure 2D), all these events often being well intertwined with one another. Although involved as apical molecular switches orchestrating virtually all cellular signaling pathways and sharing a highly similar structural arrangement, the single SFKs often demonstrate a differentiated responsiveness to VPs, which depends on the inbuilt properties of the kinase structure and the mode of interaction with VPs, especially with respect to the SH3 and SH2 domains, resulting in remarkably

Table 1 List of the viral proteins described in this review

Protein	Virus	Src family kinase	Phosphorylation sites (tyrosines)
Polyoma middle-T antigen	Polyomavirus	Src and Fyn	Y250, Y315 and Y322
Tyrosine kinase interacting protein	Herpesvirus saimiri	Lck	Y114 and Y127
Tegument protein VP11/12	Herpes simplex virus	Lck	Y not identified
Accessory viral protein X	Human immunodeficiency viruses and Simian immunodeficiency viruses	Fyn	Y66, Y69 and Y71
Latent membrane protein 2A	Epstein-Barr virus	Lyn	Y74 and Y85
Non-structural protein 5A	Hepatitis C virus	Src	Y not identified
Accessory protein Nef	Human immunodeficiency viruse-1	SFKs	None
Non-structural 1	Avian influenza virus	Src	None
Accessory protein p13	Human T-cell leukemia virus type 1	SFKs	None
RNA-dependent RNA polymerase non-structural protein 5B	Hepatitis C virus	Src	None

SFKs: Src family kinases; VP: Viral protein.

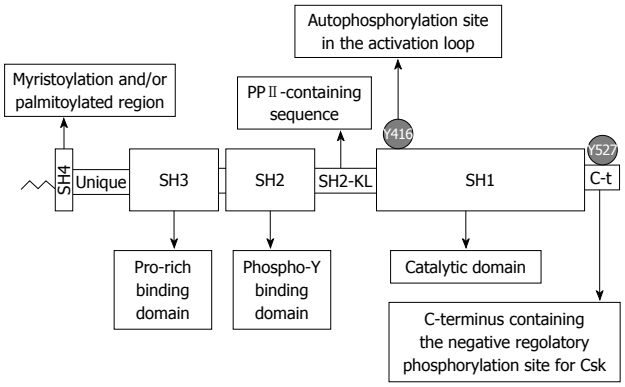

Figure 1 Diagram representing the domain organization of Src family kinases. As reported in the text, the C-terminus (C-t in the figure) when phosphorylated at Tyr527 binds to the Src homology (SH)2 domain and the polyproline type Ⅱ helical motif (PP Ⅱ) motif in the SH2 kinase linker (SH2-KL in the figure) engages the SH3 domain, thus inducing an inactive conformation. Disruption of these inhibitory interactions, in the case of viruses mostly induced by proteins bearing tyrosine phosphorylated or proline-rich motifs, leads to the full activation of Src family kinases.

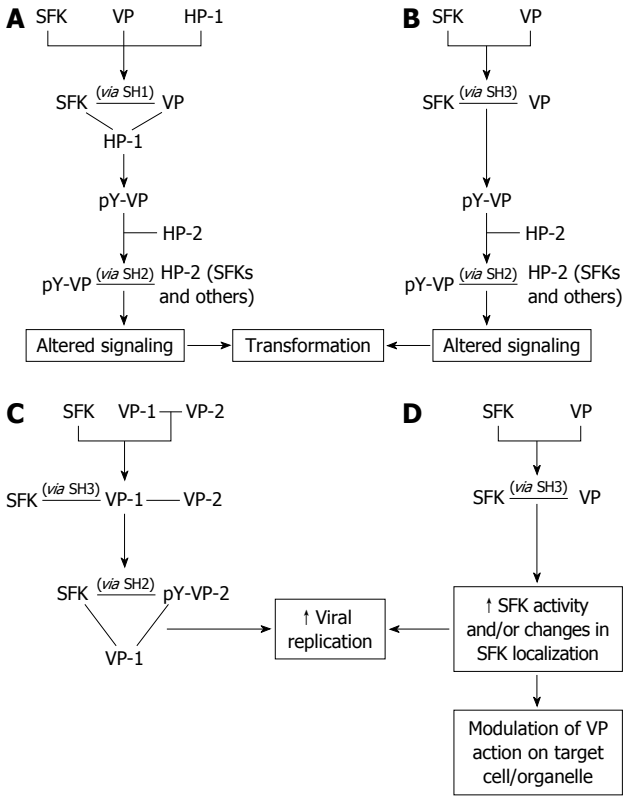

Figure 2 Models of mechanisms connecting viral proteins and Src family kinases and downstream effects. Viral proteins bind to the various modular domains of Src family kinases (SFKs), with (A) or without (B-D) concurrent association with other host proteins, resulting in the subsequent phosphorylation of the viral proteins (VPs) by which they are engaged (A, B), ultimately conferring new functional properties to VPs, or of different VPs to stabilize multiprotein complexes (C); Moreover, VPs may delocalize SFKs to different cell compartments, where SFKs in the activated form can process local substrates or act as non-catalytic mediators of the action of VPs themselves (D) (see text for further details regarding how specific VPs fit into each model. Solid lines indicate binding, arrows indicate downstream event. HP: Host protein; pY: Phosphotyrosine.

diverse effects on their non-redundant functions in a cellular scenario. This can be exemplified by VPs that can activate certain SFKs, but inhibit or leave unaffected others in the same cell type, as long as they are co-expressed. Hence, this observation, if considered in the more complex framework of the virus-host relationship, may help devise therapeutic strategies aimed at developing drugs capable of selectively disrupting VP-SFK interactions without altering signaling networks essential for the host cell life.

VPS AS SUBSTRATES FOR SFKS

The number of tyrosine phosphorylated VPs is, for the time being, rather small and these mostly include molecules from classes of viruses that can establish chronic infections, such as herpesviridae, polyomaviridae and retroviridae, among others (Table 1).

The polyoma middle-T (MT) antigen, an early product of the viral lytic cycle of the polyomavirus, which is known to contribute to the onset of multiple tumors, is phosphorylated by Src and Fyn at tyrosines that become docking sites for molecules essential for downstream signaling [*e.g.*, PLCγ, phosphatidylinositol 3-kinase (PI3K) and Shc][11]. This multiprotein complex then mimics a

constantly activated growth factor receptor at the plasma membrane. Importantly, a preliminary step for SFKs to phosphorylate the MT is their activation, which occurs upon binding of their SH1 domain to the MT itself and concurrently to the dimeric core of the serine/threonine protein phosphatase 2A, which acts as a scaffold between the MT and SFKs[12,13] (modeled in Figure 2A).

Another VP that associates to the plasma membrane and exhibits a transforming ability owing to phosphorylation by the SFK Lck is the tyrosine kinase interacting protein (Tip), an oncoprotein encoded by the genome of herpesvirus saimiri, a T-lymphotropic monkey herpes virus. Importantly, Lck phosphorylates Tip at different tyrosines responsible for binding the SH2 domains of various signaling molecules, among which are Lck itself, STAT3 and STAT6, all required for promoting the transformed phenotype[14,15]. Also in this case, the essential requirement for phosphorylation is the initial interaction between the SFK and the substrate, here mediated by Tip's sequence homologous to the C-terminus of SFKs and SH3-binding PRM with Lck[16]. The plasma membrane also serves as the anchoring site for VP11/12, a tegument protein residing between the capsid and the envelope of the herpes simplex virus immediately after viral entry, this VP being strongly phosphorylated by SFKs and hence modulating cellular signaling pathways[17]. Of note, the function of VP11/12 as a component of a signalosome differs from that exerted by VP11/12 itself in the virion assembly at the perinuclear cytoplasmic foci of the infected cells, whereby it also massively localizes. During T cell infection, VP11/12 behaves as an activator and a substrate of Lck, which phosphorylates VP11/12 at a number of tyrosines serving as docking sites for, at least as documented thus far, the SH2 domain of Lck itself and the PI3K regulatory subunit p85[18]. It is to be underlined that under these conditions Lck does not operate as a transducer of TCR signaling but triggers cellular processes peculiarly directed by VP11/12, which are still being investigated. Other aspects that remain to be explored are the mechanism of Lck activation and the mode of recognition of VP11/12 as a substrate by Lck, for which, on the basis of the examples mentioned above, an interaction between linear binding motifs in VP11/12 and the non-catalytic domains of the SFK may be required. Indeed, the primary sequence of VP11/12 exhibits several PRMs, which might provide an anchorage for the SFK-SH3 domain with subsequent structural changes from the inactive close to the active open conformation of SFKs, to the catalytic pocket of which the access of VP11/12 as substrate is then favored. That the phosphorylation of VPs and the interaction between specific motifs harbored by VPs and the modular domains of SFKs may be connected seems to be further confirmed by the findings regarding Vpx, an accessory protein of the human immunodeficiency virus type 2 (HIV-2) and the simian immunodeficiency virus[19]. This VP, which is coupled to the preintegration complex and localizes to the nuclei of infected cells, shuttles to the cytoplasm to be incorporated into the viral core and ensures efficient viral replication only if it becomes phosphorylated by the SFK Fyn (especially at Y66, 69 and Y71)[20]. This event is made possible by a preliminary interaction between Vpx PRMs and Fyn SH3 domain, which may bring Vpx to the Fyn catalytic domain[21].

The nature of the binding of other VPs that are tyrosine phosphorylated and bind to SFKs remains unclear, requiring in-depth examination. One instance may be latent membrane protein 2A (LMP2A), a VP encoded by Epstein-Barr virus genome in infected B cells, which, by sequestering proteins normally associated with the B cell receptor (BCR) in the absence of BCR-triggering antigens, mimics the latter with subsequent activation of downstream survival signaling pathways, hence altering normal B cell development[22]. In particular, it has recently ascertained that phosphorylation of LMP2A by Lyn, the most expressed SFK in B lymphocytes, exerts a dual role, namely as (1) an early requirement for the formation of the LMP2A-based signalosome through creation of docking sites for the Spleen tyrosine kinase (Syk), the SH2 domain-containing adapter protein B (Shb) and Lyn itself[23]; and as (2) a key regulatory event in the modulation of LMP2A-dependent signaling by degradation of various components of this multiprotein complex (Lyn and even LMP2A itself)[24]. Also in the case of LMP2A, it is not clear how Lyn becomes activated before phosphorylating the VP. In this respect, of the several PXXP motifs along the sequence of LMP2A, the N-terminal ones have only been tested for binding SH3 domains, proving unable to do so and thus leaving open the possibility that the C-terminal PP Ⅱ helical motif may be a site of interaction and activation for Lyn[23] (the basic mechanisms demonstrated or thought to underlie interaction and downstream effects of Tip, VP11/12, Vpx and LMP2A with SFKs are illustrated in Figure 2B).

An even more intricate case is represented by the non-structural protein 5A (NS5A) encoded by the genome of the hepatitis C virus (HCV), which is essential for HCV replication and virion assembly in hepatocytes[25]. This VP has recently been found to be phosphorylated at tyrosines within SH2-binding motifs, in addition to being highly phosphorylated at serine residues[26-28]. It also bears a conserved C-terminal PRM that has been shown to bind to the recombinant SH3 domains of Hck, Lck, Lyn and Fyn, but not Src, negatively affecting the activity of Hck, Lck and Lyn but stimulating that of Fyn[29]. Although not binding to Src *via* the SH3 domain thereof, NS5A interacts with the SH2 domain of this SFK after being phosphorylated in HCV-infected hepatocytes, an event which is critical for viral replication, and whereby Fyn, the only SFK activated by NS5A upon interaction mediated *via* SH3 domain, proves dispensable[27]. A possible mechanism for the phosphorylation of NS5A and subsequent SH2 domain-mediated interaction with Src is set forth in the next section and modeled in Figure 2C. Besides, in B cells, whereby infection by HCV causes mixed cryoglobulinemia and B cell non-Hodgkin's lymphoma, Fyn inter-

acts with NS5A through both its SH2 and SH3 domains in a tyrosine phosphorylation-dependent manner and by recognition of a PRM of NS5A itself, respectively, resulting in inhibition of viral replication in parallel with Fyn enhanced activity[28]. These data again confirm that VPs need to specifically select SFKs, whose non-redundant functions can be exploited to dictate the different steps of viral replication.

EFFECTS ON LOCATION AND ACTIVITY OF SFKS MEDIATED BY INTERACTION WITH VPS

The mechanisms leading to the phosphorylation of VPs described above seem to mainly point to the earlier binding of VPs to the SH3 domain of SFKs as the activation event for SFKs themselves, the newly phosphorylated tyrosines providing an anchorage for signaling molecules utilized by the virus for its own benefit, even for SFKs, among others. We shall hereafter illustrate a set of VPs that are still able to interact with the SH3 domain of SFKs and function as activators of SFKs without being their substrates (a general diagram is shown in Figure 2D). From this list, the well-characterized HIV-1 accessory protein Nef stands out, it being essential for virus replication and acquired immunodeficiency syndrome pathogenesis by interacting with various host cell proteins involved in immune recognition and survival, among which SFKs are targeted with high selectivity[30-32]. Among the many interaction motifs along its sequence, Nef harbors a highly conserved PxxPxR motif, which, together with a hydrophobic pocket in the core region[33], takes part in the interface between Nef and the SH3 domain of a few of SFKs, namely Hck and Lyn[33], thus causing disruption of the negative regulatory interaction between the SH2-kinase linker and the SH3 domain itself on the back of the kinase domain and subsequent activation. Of note, in spite of the high conservation of Nef's regions for binding to these SFKs, other determinants have emerged as critical in this function and also in influencing replication of HIV-1 variants. As an example, the R71T mutation occurring immediately upstream of the PRM has been correlated with a lower ability of Nef to bind SFKs as well as a decreased capability of the HIV-1 strain bearing this mutation of replicating[34]. At the cellular level, Nef activates and reroutes specific SFKs to the Golgi apparatus, the preferential subcellular localization of this VP, thereby optimizing the environmental conditions for viral replication and provoking severe alterations of the immune response. In macrophages, Nef is described as hijacking and activating Hck, localizing it to the Golgi apparatus and perturbing the N-glycosylation/trafficking processes by triggering the MAP kinase ERK-GRASP65 cascade[35,36]; instead, in T lymphocytes, Lck is directed by Nef from the plasma membrane to the trans-Golgi network, which prevents Lck from being recruited to the immunological synapse, whose altered formation in turn results in interfering with TCR signaling[37]. In contrast to Nef, whose role has been and still is being deeply explored, the pathophysiological consequences of the interaction of other VPs with SFKs are far from being totally clarified.

The non-structural 1 (NS1) protein of the avian influenza virus (AIV), a multifunctional protein with interferon-antagonistic properties, is a further example of the interaction between the SH3 domain of SFKs through PRMs of specific VPs as a means to mediate pathogenicity by viruses[38]. First being isolated in poultry and having exhibited high virulence and pathogenicity, it was also shown to cross the species barrier, involving human fatalities, especially in the Far East. The possibility that such a viral strain or new reassortants might cause severe pandemics generated a new interest in evaluating the pathogenicity determinant in the spread and pathogenesis of the disease[39,40]. NS1 possesses two functional domains, the N-terminal RNA-binding domain containing one nuclear localization signal as well as a SH2- binding motif targetable after phosphorylation of the tyrosine residue, and the C-terminal effector domain, with two PRMs, a further nuclear localization sequence and a PDZ binding motif. Of the PRMs, the first is generally conserved in all influenza genotypes and harbors the structural determinants for binding the SH3 domain of the PI3K regulatory subunit p85 (PI/LPxxP)[41], whereas the C-terminal has a certain variability that parallels its capability of interacting with the SH3 domain of and activating the SFK Src[42]. In this respect, only NS1 bearing the consensus sequence type 2 for binding SH3 domains of Src (PXXPXK/R), was able to enhance the SFK activity, which occurred in virus genotypes that caused the most severe human influenza pandemics in 1918 and killed turkeys in Italy in 1999 with heavy economic losses, whereas the viral strains that were mutated in this region did not affect SFK activity. To date, the function of an activated form of Src in AIV-infected cells is not fully clear, although it is thought that it may be related to the localization of the Src-NS1 complex.

Another prototype for the change in subcellular localization of SFKs induced by VPs is represented by the human T-cell leukemia virus type 1 accessory protein p13, although the data refer to experimental approaches *in vitro* or in cultured cells transfected with the single SFKs or p13 itself. p13 is known to localize to mitochondria thanks to its N-terminal mitochondrial localization signal, where it brings about an inward K^+ current across the inner mitochondrial membrane, leading to swelling, depolarization and increased respiratory chain activity[43]. Recently, p13 has been found to bind to the SH3 domain of SFKs by a well-defined C-terminal PRM and to act as a carrier for SFKs themselves into mitochondria, this new localization of SFKs resulting in (1) a sharp rise in intramitochondrial tyrosine phosphorylation; and (2) a significant mitigation of p13's aforementioned effects on mitochondria[44]. This observation seems to be in line with recent findings that strongly suggest a new role for SFKs as factors that help preserve mitochondrial structural and functional integrity under stressful conditions[45], thus further providing

novel insights into the catalytic and non-catalytic role of SFKs in virus-infected cells[46,47]. The complex relationship between such functions of SFKs and the physical interaction thereof with VPs fits into another model represented by the events that lead to the formation of the HCV replication complex, in which the RNA-dependent RNA polymerase NS5B and the above mentioned substrate for SFKs, NS5A, together with the SFK Src, take part[27]. The integrity of this multimolecular complex, which is essential in HCV replication, requires Src as a scaffold for promoting a tighter interaction between NS5A and NS5B, with both the modular SH2 and SH3 domains as well as the catalytic activity of Src being implicated. Interestingly, Src is the only SFK that does not bind to NS5A through its SH3 domain[26], whereas it recognizes the C-terminal proline-rich region containing a non-canonical SH3 binding motif within NS5B[27]. On the other hand, the SH2 domain of Src interacts with a yet-to-be-identified tyrosine phosphorylated binding motif within NS5A, respectively. Although the molecular mechanism underlying the formation of the complex is unknown, it is tempting to speculate that Src stabilizes the weak interaction between NS5A and NS5B by firstly recognizing the PRM of NS5B, although it does not display an optimal consensus for binding to the SH3 domain of SFKs, thus becoming activated and enabled to phosphorylate NS5A. Phosphotyrosines on NS5A can then be targeted as docking sites by Src, further strengthening the stability of the heterotrimer (Figure 2C). What we hereby again underscore is that VPs and cellular proteins, such as SFKs, complexed into a new operative unit can serve as a key to interpreting the intricate relationship between host cells and viruses in order to elaborate novel strategies to disrupt aberrant multiprotein associations.

CONCLUDING REMARKS AND PERSPECTIVES

The significance of SFKs as critical mediators in the life cycle of viruses has been widely shown by the effects of the inhibition of their enzymatic activity or of their expression as well as by those related to the interaction with specific VPs, as hereby briefly reviewed. Indeed, this latter issue has become a new important field of investigation, with great efforts aimed at dissecting the structural aspects that favor such interactions, in order to develop therapeutic strategies capable of disrupting them to hamper viral replication. Although the different domains of SFKs exhibit various potential anchorage sites for VPs, including the SH2 and the catalytic domain, these preferentially target the SH3 domain of SFKs by their PRMs (usually a class Ⅱ motif), which generally displays the consensus sequence PxΦPxK/R (where Φ stands for a hydrophobic residue). The PRM-bearing protein can then compete with and displace the PPⅡ within the SH2-kinase linker of SFKs, thus directly interacting with the SH3 domain and inducing the transition from the "closed" to the "open" conformation, with three possible outcomes: (1) altered localization of SFKs; (2) phosphorylation of the VP with generation of docking sites available for further interactions; and (3) hyperactivation of the kinase activity. This latter effect has been explained for Hck bound to Nef, but not thus far for other SFKs, through kinetic studies, which have highlighted that Nef's PRM induces a change in the conformation of the active site of Hck by an allosteric mechanism with a decrease in the K_M for ATP[48]. Of note, even if all SFKs share a common domain structure and a mode of regulation, VPs may display different abilities to interact with each single SFK and to affect their catalytic activity by their PRM. This wide variability in the response of the single SFKs demands a further effort to extend our knowledge on the structural determinants of the SH3 domain also outside of the interface binding the viral PRM, this possibly providing the ground for the prediction of recognition elements. From a therapeutic perspective, two main schools of thought have surfaced in addressing this issue, one arguing for the use of bioengineered polypeptides capable of interfering with SH3 binding of SFKs to VPs[49-51] and the other supporting the implementation of non-toxic kinase inhibitors that bind the catalytic groove of SFKs only if the VP:SFK complex is formed (*e.g.*, Nef:Hck)[52,53]. This latter approach would be remarkably useful in preventing unpleasant or harmful side effects, since such drugs would not affect the pool of uncomplexed SFKs involved in other cellular activities.

The data hereby summarized lead us to assume that, despite the widespread occurrence of PRMs on VPs and the existence of over 200 SH3 domains, PRMs are directed to specific host targets, among which SFKs are crucial actors in sustaining virus survival[54-56]. Besides, the interaction between SFKs and PRMs of VPs seems to be emerging as a novel issue of special interest in the light of their association with virulence of viral strains and the level of pathogenicity, as reported for AIV NS1[42] and HIV-1 Nef[33,34], with the enhancement of the kinase activity being a sort of epiphenomenon related the severity of disease. In this respect, retrospective studies on highly pathogenic virosis and structural analysis of PRM-bearing proteins as well as their effect on SFKs would open new perspectives and provide further hints for pharmaceutical research and clinical applications.

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TOPIC HIGHLIGHT

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Viral manipulation of cellular protein conjugation pathways: The SUMO lesson

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Abstract

Small ubiquitin-like modifier (SUMO)ylation is a key post-translational modification mechanism that controls the function of a plethora of proteins and biological processes. Given its central regulatory role, it is not surprising that it is widely exploited by viruses. A number of viral proteins are known to modify and/or be modified by the SUMOylation system to exert their function, to create a cellular environment more favorable for virus survival and propagation, and to prevent host antiviral responses. Since the SUMO pathway is a multi-step cascade, viral proteins engage with it at many levels, to advance and favor each stage of a typical infection cycle: replication, viral assembly and immune evasion. Here we review the current knowledge on the interplay between the host SUMO system and viral lifecycle.

Key words: Virus; Small ubiquitin-like modifier; Small ubiquitin-like modifier-ylation; Exploitation; Virus assembly; Immune evasion; Innate immunity

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INTRODUCTION

Pathogenic organisms possess the remarkable ability to hijack the cellular machinery of host cells to their advantage. Viruses in particular manipulate several physiological cellular pathways to prevent antiviral responses and to create an environment that facilitates their survival and propagation.

A common strategy to create a more conducive milieu to viral development consists in exploiting cellular post-translational modifications (PTMs) mechanisms.

Between the numerous PTMs occurring in cells, small ubiquitin-like modifier (SUMO)ylation is emerging as a key PTM that controls the function of a plethora of proteins and biological processes. Hence, given its central regulatory role, the SUMOylation pathway is widely exploited by viruses, whose proteins can either modify and/or be modified by the SUMOylation system with various consequences.

The aim of this review is therefore focused on the mechanisms through which viruses exploit the SUMOylation pathway and the implications for viral infections and diseases. First, we will describe the general characteristics of the SUMO cycle and the enzymes involved in this pathway. Next, we will give a comprehensive overview on the latest findings on viral proteins and SUMOylation interplay, focusing in particular on the mechanisms that can promote viral infections by altering the SUMO system.

SUMO PATHWAY

The SUMO is a member of the ubiquitin-like proteins (Ubls) family. The common feature of Ubls is that they

are attached to a target protein amino group of a lysine residue through similar but distinct enzymatic cascades[1]. After conjugation, Ubls reversibly alter protein functions, without the need for new protein synthesis, thus providing cells with a rapid and versatile mechanism to quickly respond to changes in the surrounding environment.

SUMOylation was identified as a reversible PTM in 1996[2,3]. There are four different genes in the human genome coding for the different SUMO modifiers: SUMO-1, -2, -3 and -4. SUMO-2 and -3 are nearly identical in sequence, differing from each other by only three N-terminal residues, and are therefore collectively referred to as SUMO-2/3[4]. On the contrary, SUMO-2/3 significantly diverge from SUMO-1, sharing only about 45% similarity with its sequence[5]. Finally, a SUMO-4 isoform has been described[6], which shares 86% homology with SUMO-2. In humans, while SUMO-1 and SUMO-2/3 are ubiquitous, SUMO-4 expression seems to be restricted to kidneys, lymph nodes and spleen[6].

SUMO-1 conjugation has been implicated in the regulation of physiological processes because it is virtually all bound to target proteins, while SUMO-2/3 appears to be more widely expressed as a pool of free non-conjugated proteins, readily available for stress responses[4]. SUMO-4 is probably not conjugated under normal conditions and its biological role is still unclear[7]. Moreover, the different SUMO paralogs do preferentially conjugate some substrates[4,8,9], although other proteins can be equally modified by SUMO-1 or SUMO-2/3[8,10,11].

SUMO attachment to target proteins is mediated by enzymatic reactions (schematized in Figure 1) that catalyze the formation of an isopeptide bond between the SUMO C-terminus and the ε-amino group of an internal lysine in the target, generally but not necessarily found within a SUMO modification consensus motif, ψKxE[12,13] (where ψ is a bulky aliphatic residue, X is any residue).

Interestingly, SUMO-2/3 also bear the ψKxE motif and therefore can be SUMOylated, forming chains on substrate proteins through their internal lysine residue[14]. Although the formation of SUMO-1 chains has also been observed both *in vitro* and *in vivo via* non canonical consensus sites[15-17], usually SUMO-1 acts as terminator of SUMO-2/3 polymeric chains[15]. Although target proteins are predominantly conjugated to monomeric SUMO, SUMO chains also play roles in replication, turnover of SUMO targets, mitosis and meiosis[18].

SUMO proteins are 11 kDa and, similarly to most other Ubls, are synthesized as inactive precursor proteins carrying an extension of variable length (ranging from 2 to 11 amino acids). These primary translated products undergo a C-terminal cleavage to expose the diglycine motif that will be linked to the target proteins. Removal of this C-terminal end is mediated by a specific protease belonging to the sentrin-specific proteases (SENPs) family[19]. In addition to its role in SUMO processing, SENP activity is also required for SUMO depolymerization and deconjugation from its substrates[19], as detailed below.

The mature form of SUMO is conjugated to the target

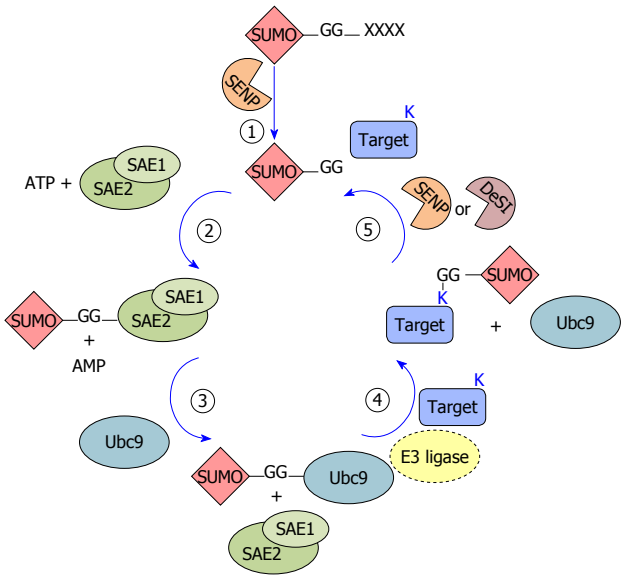

Figure 1 Schematic representation of the small ubiquitin-like modifier conjugation enzymatic cascade. The cartoon schematically represents the enzymatic steps of small ubiquitin-like modifier (SUMO) protein conjugation on lysines of substrate proteins. 1: SUMO protein precursors are processed by SUMO proteases (SENP) that remove the C-terminal tetrapeptide (X) and free the diglycine motif (-GG); 2: The mature form of SUMO is activated by adenylation at the C-terminal diglycine motif by the E1 enzyme (the SUMO activating enzyme, SAE1-SAE2 or AOS1-UBA2) promoting a thioester bond with a conserved Cys of the E1 enzyme; 3: SUMO is then transferred to a Cys on the E2 conjugating enzyme (Ubc9) forming an E2-SUMO thioester; 4: An isopeptide bond is formed between the diglycine motif of SUMO and a lysine (K) residue in the substrate. E3 ligases are dispensable *in vitro* but most likely required *in vivo*; 5: SUMO proteins are removed from substrates by the action of SUMO proteases (SENPs) or DeSumoylating-isopeptidase (DeSI) and free SUMO proteins are available for another cycle of conjugation.

proteins by a three-step enzymatic cascade, very similar to the ubiquitin pathway but involving different enzymes: E1 activating enzyme, E2 conjugating enzyme and E3 ligases (Figure 1).

SUMO E1 is a 110 kDa protein, composed of a heterodimer of SUMO-activating enzyme subunit (SAE) 1/2 subunits (also known as AOS1-UBA2[20,21]). During each conjugation cycle, SAE1/2 activate SUMO proteins[20] through the formation of a high-energy thioester bond between SAE2 and the C-terminal portion of SUMO[22]. Activated SUMO is then transferred[22] to the E2 enzyme ubiquitin-conjugating 9 (Ubc9). Opposite to the ubiquitin pathway, where numerous conjugating enzymes have been described, Ubc9 is the only known SUMO-conjugating enzyme[23,24] and is essential for viability in most eukaryotes[25]. Although Ubc9 itself can transfer SUMO to targets[26], specific SUMO E3 ligases are required for efficient modification.

SUMO E3 ligases can be classified into three groups on the basis of their similarity to the ubiquitin E3 ligases and in their mechanism of action, but they share the ability to act as a bridge between the Ubc9-SUMO complex and the target protein, functioning as substrate recognizers[27]. The first group encompasses members of the protein inhibitor of activated STAT (PIAS) family (PIAS1, PIAS3, PIASxα, PIASxβ and PIASy, reviewed in[28]). In

addition to the PIAS proteins, other secretory protein (SP)-RING domain-containing proteins function as SUMO E3 ligases (TOPORS[29], MUL1[30] and MMS21[31]). All these members contain a RING domain (SP, Siz/PIAS-RING) similar to the one found in ubiquitin E3 ligases. The second group is represented exclusively by the nucleoporin RanBP2 that seems to act as a composite E3 ligase in the RanBP2/RanGAP1*SUMO1/Ubc9 complex[32]. The third group comprises E3 ligases lacking the RING-domain such as the polycomb member Pc2[33], histone deacetylase (HDAC)4[34], HDAC7[35], the G-protein Rhes[36], the RNA-binding protein translocated in liposarcoma[37] and tumor-necrosis-factor-associated protein 7[38]. Moreover, members of the diverse tripartite motif (TRIM) family have been very recently discovered as a new group of SUMO E3 ligases, requiring TRIM (defined by a RING domain, one or two zinc-binding domains and a coiled-coil dimerization region) to stimulate the conjugation of both SUMO-1 and SUMO-2/3 to target proteins[39,40].

SUMOylation is a reversible process, governed by SUMO-specific proteases belonging to the SENP family and by the recently found DeSumoylating-isopeptidase (DeSI) proteins. Six true human SENP proteins have been described so far (SENP1, 2, 3, 5, 6, 7), differing in their cellular distribution, selectivity for SUMO maturation and deconjugation towards different SUMO paralogs[41]. SENP1 and SENP2 are specific for both SUMO-1 and SUMO-2/3 processing and deconjugation, while SENP3 and SENP5 act preferentially on SUMO2/3. SENP6 and SENP7 seem involved mainly in deconjugating SUMO2/3 chains (see[41] and citations therein). Finally, SENP8 shows substrate specificity to another Ubl, NEDD8[42]. All the SENPs localize to the nucleus or nucleus-associated structures; on the contrary, DeSI (-1 and -2) proteins localize also in the cytoplasm and show deSUMOylating but not processing activity for SUMO1 and for both monomeric and polymeric SUMO2/3 chains[43].

Most cellular SUMO targets are transcription factors and usually SUMOylation exerts an inhibitory effect on their transactivating activity[44], by sequestering the transcription factor in ProMyelocyticLeukemia nuclear bodies (PML-NBs)[45], a nuclear domain whose assembly requires an active and efficient SUMOylation pathway[46].

Usually, after undergoing SUMOylation, the substrate protein is recognized by a binding partner containing a SUMO-interaction motif (SIM)[47]. This interplay can lead to an altered binding with interacting proteins or DNA, promotes the recruitment of another SIM-containing effector, and affects the stability, localization or enzymatic activity of the SUMOylated protein. Through these mechanisms, SUMOylation regulates a number of cellular processes, such as transcriptional regulation, mRNA maturation, meiosis, mitosis, chromatin remodeling, ion channel activity, cell growth and apoptosis (reviewed in[48]).

Therefore, because of SUMOylation marked involvement in the regulation of cell functions, it is easy to understand why viruses have evolved a variety of mechanisms to exploit this system to their advantage.

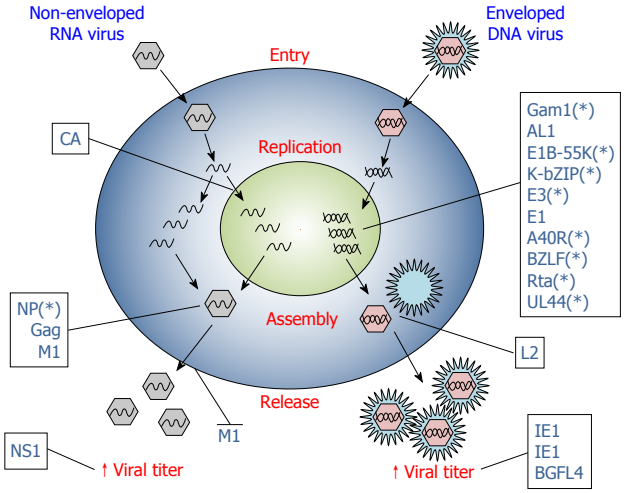

Figure 2 Viral proteins exploit small ubiquitin-like modifier at different steps of virus lifecycle. Scheme representing the different stages of viral infection (entry, replication, assembly, release) in host cells. Viral proteins interact with the small ubiquitin-like modifier (SUMO) machinery to promote different steps of viral life cycle, as represented. Viral proteins are designed with their acronym. The asterisk (*) indicates that the marked viral protein has not been formally shown to directly influence viral life cycle by exploiting SUMOylation, but indications of a mechanistic link are known. See text for further details on exploitation of the SUMO machinery by single viral proteins.

MANIPULATION OF THE SUMO PATHWAY: A VERSATILE SWITCH TO PROMOTE VIRAL LIFESPAN

Many different viral proteins have been characterized for their ability to interact with the SUMO pathway. Since the SUMO pathway is a multi-step cascade, viral proteins can interact with and exploit it at many levels, in order to promote each stage of a typical infection cycle (Table 1). Indeed, viruses can utilize the SUMO cellular machinery to support viral persistence and replication, assembly of the virus, and to avoid the host immune system.

In the following sections we will detail some examples of the current knowledge about the interplay between SUMO dysregulation and viral lifecycle.

SUMOylation and viral replication

Viruses lack some of the components required to replicate their genetic material and therefore need to redirect cell activities to promote their own reproduction. A large body of evidence supports a role for SUMOylation in replication of many viruses. In particular, viruses can subvert the transcriptional profile or the proliferation activity of the host cell, dysregulate the host cell cycle or interfere with the apoptotic process, or exploit SUMOylation to regulate the transcriptional activity of viral proteins involved in virus replication (Figure 2). Here we will describe only some illustrative examples of the strategies used by viruses to promote their own replication using the SUMOylation machinery. In fact, there are many other viral proteins known to interact with and/or modify the SUMO pathway and in many cases the bio-

Table 1 Viral life cycle and small ubiquitin-like modifier pathway

Virus	Protein	Interaction with SUMO	Proposed impact on viral or cellular activity	Ref.
Chicken embryo lethal orphan Adenovirus	Gam1	Overall SUMOylation decrease through SAE1/2 degradation and reduced Ubc9 expression	Cellular transcriptional activation	[49,50]
			PML-NBs dispersion	[51]
		Interference with HDAC1 SUMOylation	Cellular transcriptional activation	[51]
Geminivirus	AL1	Promotes SUMOylation of selected host factors	Viral replication	[57,58]
Adenovirus	E1B-55K	E3 ligase SUMO-1 specific	Regulator of cell cycle and apoptosis	[59,60]
		SUMOylated	Cellular transformation	[63]
			Interaction with PML-NBs	[62,64]
Kaposi's sarcoma-associated herpesvirus	K-bZip	E3 ligase SUMO-2/3 specific	Regulator of cell cycle and apoptosis	[66]
		SUMOylated	Cellular transcriptional repression	[65,66]
	K-Rta	E3 ubiquitin ligase activity against SUMOylated proteins	PML-NBs dispersion	[119]
Vaccinia	E3	SUMOylated	Regulator of apoptosis	[69]
	A40R	SUMOylated	Viral replication	[74]
Bovine papillomavirus	E1	SUMOylated	Viral replication	[71,72]
Human papillomavirus	L2	SUMOylated	Viral capsid assembly	[93]
		Increase in cellular SUMO 2/3 conjugation		[93]
Moloney murine leukemia	CA	SUMOylated	Viral replication	[75]
Influenza A	NS1	SUMOylated	Viral replication	[78]
Cytomegalovirus	IE1	SUMOylated	Viral replication	[80,81]
		Interaction with selected host factors SUMOylated	PML dispersion	[123]
	IE2	Interaction with SUMOylated proteins	Viral replication	[82]
		SUMOylated	Viral replication	[83]
	UL44	Interaction with Ubc9	Viral replication	[84]
Epstein-Barr	BZLF1	SUMOylated	Reactivation of latent infections	[85,86,88]
	Rta	SUMOylated	Reactivation of latent infections	[89]
	BGLF4	Interaction with SUMOylated proteins	Viral replication and reactivation of latent infections	[91]
			PML-NBs dispersion	
Hantaan	NP	Interaction with Ubc9 and SUMO-1	Virus assembly	[95,96]
Mason-Pfizer; human immunodeficiency	Gag	Interaction with Ubc9	Virus assembly	[97] [98-100]
Influenza A	M1	SUMOylated	Virus assembly	[102,103]
Ebola Zaire	VP35	Promotes SUMOylation of selected host factors	IFN inhibition	[109,111]
Herpes simplex type-1	ICP0	E3 ubiquitin ligase activity against SUMOylated proteins	PML-NBs dispersion	[115-117]
Varicella zoster	ORF61	Interaction with SUMOylated proteins	PML-NBs dispersion	[120,121]
Encephalomyocarditis	3C	Interaction with selected host factors	PML degradation	[122]

The table schematizes known viral proteins interacting with the small ubiquitin-like modifier (SUMO) pathway, with a brief description of their relationship with the SUMOylation machinery and the proposed biological outcome. References are also reported (see text for further details). SAE: SUMO-activating enzyme subunit; HDAC: Histone deacetylase; PML-NBs: ProMyelocyticLeukaemia nuclear bodies; IFN: Interferon.

logical significance of this interplay is still obscure.

A hallmark of viral infection is the increase of host transcription to sustain viral replication. Gam1 is an early gene expressed by chicken embryo lethal orphan (CELO) Adenovirus that has the remarkable ability to inhibit global SUMOylation. Work from our group demonstrated that Gam1 decreases the overall SUMOylation by interaction and consequent degradation of the E1 heterodimer[49,50]. Specifically, Gam1 recruits both SAE1 and SAE2 into Cul2/5-EloB/C-Roc1 ubiquitin ligase complexes and subsequently targets SAE1 for ubiquitylation and degradation. SAE2 depletion is not tightly related to Gam1, but is rather an effect of SAE1 disappearance[50].

We also observed that Ubc9 levels are reduced upon Gam1 expression, by a yet undefined mechanism[49]. Furthermore, Gam1 disperses PML-NBs concomitant to a strong loss of SUMO-1 from the nucleus[51]. As SUMO conjugation to many transcription factors represses their activities[48], the overall decrease in SUMO conjugation caused by Gam1 could increase cellular transcriptional activity, which in turn could facilitate viral replication. Gam1 also interferes with SUMOylation of endogenous proteins such as HDAC1[51]. HDAC1 SUMOylation has an impact on the transcriptional repressive potential of the deacetylase[52,53]. Moreover, HDAC1-containing chromatin remodeling complexes are known to be exploited by viruses to regulate the progression of their infection[54]. Interestingly, a replication deficient ΔGam1 CELO virus[55] can be rescued by HDAC inhibitors treatment[56], suggesting the existence of a cross talk between cellular SUMOylation and acetylation that can be subverted and exploited by Gam1, an essential gene for CELO replication[55].

While Gam1 promotes broad changes in the global SUMOylation pattern of the host cell, AL1 protein encoded by the plant pathogen Geminivirus alters the SUMOylation status of only selected proteins[57]. AL1 is the only plant pathogen protein described so far as interacting with the SUMO pathway[58], by associating with

the plant E2 conjugating enzyme SCE1. As mentioned, AL1-SCE1 complex in plants does not produce an overall alteration of host proteins, but seems to modulate the SUMOylation level of selected host factors to create an environment suitable for viral infection.

E1B-55K is an Adenoviral early protein that functions as an E3 SUMO ligase that specifically conjugates SUMO-1 but not SUMO-2/3 to p53, inhibiting its transcriptional activity[59]. Indeed, expression of E1B-55K protein induces p53 SUMOylation[60] and E1KB-55K/p53 co-localization to PML-NBs, thus restricting p53 nuclear mobility in living cells[59]. p53 sequestration in PML-NBs seems to be a prerequisite to the altered p53 localization and activity observed in Adenovirus infected cells, preceding and addressing its ubiquitin-proteasome dependent degradation in cytoplasmic aggresomes[61]. Notably, E1B-55K associates with PML-NBs at early times after infection[62], as does p53[61]. Hence, since p53 is one of the most recognized regulators of cell cycle arrest and apoptosis, the p53-SUMO-1 conjugation could be a key event in the oncogenic transformation of primary cells induced by Adenoviruses. Interestingly, E1B-55K is also itself SUMOylated by all SUMO paralogs in a phosphorylation-dependent mechanism[63] and both SUMOylation[64] and phosphorylation[63] are required for its activity. In addition, the recent findings that E1B-55K itself interacts with Ubc9 strongly highlight that this viral protein extensively cooperates with the SUMO pathway to promote Adenovirus lifespan[63].

The K-bZIP protein encoded by Kaposi's sarcoma-associated herpes virus (KSHV) is another viral protein that utilizes the SUMO pathway to alter the host cell cycle. K-bZIP is a strong transcriptional repressor whose activity, similarly to E1-55K, depends on SUMOylation[65], catalyzed by K-bZip itself[66]. Other similarities to the adenoviral protein include the PML-NBs localization[65] and the ability to recruit p53 to PML-NBs[67]. Finally, K-bZIP also exhibits E3 SUMO ligase activity but, unlike E1-55K, shows preferential selectivity towards SUMO-2/3 paralogs[66]. Notably, p53 SUMO-2/3 conjugation catalyzed by K-bZIP enhances p53 transcription factor ability, suggesting a p53-mediated growth arrest by prolongation of the G1 phase of the cell cycle[66]. Growth arrest is a common outcome of herpes viruses infection[68], that poses the cell in a specific phase of the cell cycle, encouraging viral replication and protecting the host cells from undergoing apoptosis.

An additional viral protein that takes advantage of the SUMO pathway to regulate the cellular apoptotic process is the E3 protein encoded by Vaccinia virus. Indeed, recent findings[69] demonstrate that E3 SUMO-1 or SUMO-2 modification has a negative effect on E3 transcriptional transactivation of the p53-upregulated modulator of apoptosis and APAF-1 genes. Therefore, these results could indicate that SUMO conjugation is a negative regulator of the transcriptional activation of p53 by E3.

Also, bovine papillomavirus (BPV) E1, the major initiator protein for BPV replication[70], is SUMO modified but, opposite to Vaccinia virus E3, only by the SUMO-1 paralog[71]. This covalent modification is required for E1 intranuclear localization and influences viral replication activity[72].

A40R is another Vaccinia virus protein that interacts with the SUMO system to accomplish its function. Vaccinia are unique among DNA viruses because DNA replication occurs entirely in discrete cytoplasmic structures enveloped by endoplasmic reticulum (mini-nuclei) membrane, rather than in the nucleus of the infected host cell[73]. A40R gene product is SUMO-1 modified, but unlike what has been described so far, this modification appears to be very stable and not subjected to SENP deconjugating activity[74]. Consistently, all other viral proteins SUMO-1 modified are localized into the nucleus, while A40R-SUMO-1 expression has been found in the cytosolic side of endoplasmic reticulum, the same membranes that wrap the virus replication sites. The specific localization of A40R strongly suggests a role for SUMOylation in Vaccinia replication[74].

Also, Moloney murine leukemia retrovirus capsid protein (CA) utilizes a similar mechanism. In fact, this protein interacts simultaneously with both Ubc9 and E3 ligase PIASy[75], resulting in covalent transfer of SUMO-1 to CA. Surprisingly, suppression of SUMO-1 attachment by CA mutations at Ubc9 or PIASy binding sites blocks virus replication *in vivo*, but does not affect late stages of viral gene expression or virion assembly[75]. On the contrary, Rous sarcoma virus (RSV) CA-Ubc9 interaction and SUMO-1 conjugation does not influence RSV replication[76].

Nonstructural protein 1 (NS1) is one of the major factors involved in Influenza A virus replication[77]. NS1 is able to interact with human Ubc9 and is preferentially modified by SUMO-1[78]. This characteristic seems to be conserved among most Influenza virus strains, underlining the importance of SUMO modification in Influenza virus infection. SUMO-1 modification enhances the stability of NS1 and its ability to suppress host protein expression causing an acceleration in viral replication rate[78].

Cytomegalovirus (CMV) immediate early 1 (IE1) is a viral protein that acts as a key regulator of early events in virus infection cycle together with IE2. While IE2 activates a wide range of viral and cellular promoters, IE1 only modestly promotes both cellular and viral transcription[79]. However, SUMO modification of IE1 contributes to efficient CMV replication by enhancing the expression of IE2 mRNA derived from the same transcription unit, by a yet unidentified post-translational mechanism[80,81]. Furthermore, IE2 is also SUMOylated by both non-covalent and covalent SUMO-modification[82]. IE2 SUMOylation is necessary for the function of this viral transcription factor and for human CMV replication[82], opposite to the activities of most transcription factors that are regulated in a negative manner by SUMO attachment[44]. Importantly, IE2 also contains a SIM motif to interact with other SUMOylated partners, such as TAF12, a component of the transcription factor IID complex[83]. This interaction enhances the transactivation activity

of IE2, playing a further role in the progression of the CMV lytic cycle[83].

Recently, Loregian's group reported the first evidence of SUMOylation of a viral DNA-polymerase processivity factor: the UL44 protein from human CMV[84]. UL44 strongly binds to cellular Ubc9 and is widely SUMOylated during CMV infection, with accumulation at a later time post-infection. Interestingly, UL44 SUMOylation is dependent on its correct dimerization and proper DNA binding. CMV infection in cells overexpressing SUMO1 protein results in increased viral replication and viral titer, as well as a faster relocalization of UL44 from replicative foci, suggesting that UL44 SUMOylation could perhaps support later functions important for viral propagation[84].

Finally, other interesting examples of viral proteins SUMO-modified are Epstein-Barr virus (EBV), BZLF1 (also known as Zta), Rta and BGLF4 proteins.

EBV is usually maintained under latent conditions in B lymphocytes and to proliferate it must enter the lytic cycle driven by BZLF1 and Rta. BZLF1 is post-translationally modified by both SUMO-1[85] and SUMO-2/3[86]. BZLF1 is a transcriptional activator involved in the reactivation of EBV[87], allowing its switch from latent to lytic stage, characteristic of the EBV infection cycle. SUMOylation of BLZF1 plays a key role in this mechanism, negatively affecting its transcriptional activity. In fact, SUMOylated BLZF1 associates with HDAC3 and this association allocates HDAC3 to BLZF1-responsive promoters, repressing the transcription of BLZF1-induced genes[88]. Furthermore, the SUMO-mediated repression of BLZF1 is reverted by the action of a specific protein kinase (EBV-PK) that, by inhibiting BLZF1 SUMO-conjugation, promotes the transcription of BLZF1 target genes and replication of the viral genome[86].

Similarly, also Rta SUMOylation, mediated by the adaptor cellular protein RanBPM, enhances its transactivation activity and promotes viral replication of the latent EBV virus[89].

BGLF4 is a protein kinase that phosphorylates both viral and host proteins[90], strongly contributing to the EBV infection cycle. BGLF4 carries SIM motifs responsible for its binding to SUMO-2 conjugated proteins. The SUMO binding function of BGLF4, among others, is also required to enhance the production of extracellular virus during EBV lytic replication and to disperse PML-NBs[91]. Indeed, BGLF4 seems to inhibit SUMOylation, thus promoting activation of the EBV BZLF1 protein (see above), probably by SIM-mediated recruitment and phosphorylation on SUMOylated BZLF1[91].

SUMO and virus assembly

Virus assembly is the result of a series of protein-protein and protein-lipid interactions that permits localization of different viral components at sites of virus budding. Although specific for each virus strain, virus assembly typically involves the expression of late genes that direct capsid assembling and enveloping. Besides its key role in the activity of the early expressed viral proteins that drive viral replication, SUMOylation also plays fundamental roles in viral assembly processes (Figure 2).

L2, together with L1, is a structural protein of the human papillomavirus (HPV) capsid critical for the generation of infectious viral particles as well as in early events of HPV infection[92]. L2 is preferentially modified by SUMO2/3, affecting its stability[93]. In fact, SUMOylated L2 has an increased half-life compared to the non-SUMOylated mutant. Moreover, the effect of SUMOylation negatively affects L2 capacity to interact with its physiological interactor L1, suggesting a mechanism by which capsid assembly may be modulated in HPV infected cells[93]. Moreover, L2 also increases the overall SUMO-2/3 conjugation of host proteins[93].

Nucleocapsid protein (NP) of Hantaan virus (HTNV) is a structural protein that, through its oligomerization and ability to bind RNA[94], is involved in viral assembly in the infected cell. Ubc9 and SUMO-1 interaction with NP[95] determines its localization at the perinuclear region where viral replication occurs[96] and, therefore, could regulate the assembly of the HTNV. Notably, Ubc9 was also identified as a cellular protein that interacts with the Gag protein of Mason-Pfizer monkey[97] and Human Immunodeficiency viruses[98-100], regulating viral assembly, trafficking and Env incorporation. However, these activities are not dependent on Ubc9 conjugation activity, indicating that SUMOylation may not be strictly required for assembly of these viruses[97,99].

A large body of evidence shows that Influenza A virus M1 protein is essential for viral assembly and budding[101]. M1, together with other viral proteins are SUMOylated during Influenza virus infection[102,103]. Moreover, abolishment of M1 SUMOylation resulted in dramatic reduction of the virus titer in the culture fluid, accompanied by accumulation of intracellular viral proteins and viral RNA, indicating that SUMOylation of M1 modulates the assembly of Influenza A virus. On the other hand, other steps of the viral life cycle, such as virus entry, RNA replication and translation, are not affected by M1 SUMOylation[103].

SUMOylation and viral host immune evasion

Multicellular organisms normally fight infections *via* their immune system. The immune system recognizes and combats invading foreign agents through two main canonical pathways, the innate and adaptive immunity. In addition, intrinsic cellular defenses are also employed by the host to clear viral infections[104]. Intrinsic resistance represents the first line of intracellular antiviral defense that employs the classical pathogen recognition receptors (PRRs), shared by the innate immunity response, to sense viruses and to rapidly produce antiviral molecules in order to limit the initial stages of infection. Consequently and not surprisingly, viruses have evolved a variety of mechanisms to overcome cellular defenses and SUMO represents one of the most exploited pathways to this end (Figure 3).

The intrinsic and innate immune responses are primed by the activation of PRRs, such as the toll-like receptors and retinoic acid-inducible gene I which, in turn, lead to

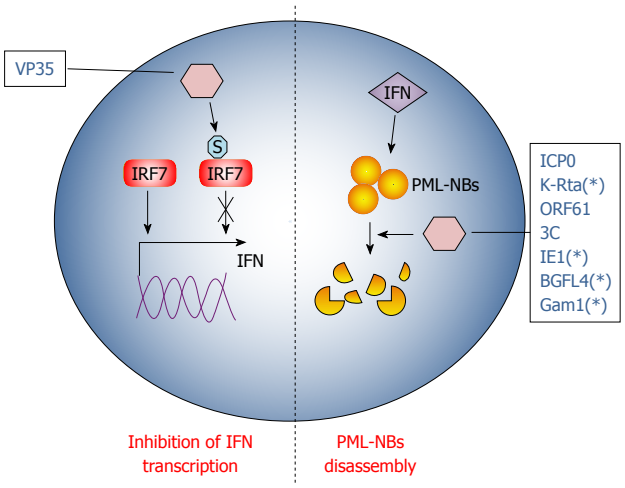

Figure 3 Viral proteins exploit small ubiquitin-like modifier to promote immune escape from innate and intrinsic responses. Schematic representation of the strategies used by viral proteins to counteract host innate and intrinsic responses through small ubiquitin-like modifier (SUMO). Viral proteins are designated with their acronym. S stands for SUMO. The asterisk (*) indicates that the marked viral protein has not been formally shown to directly influence cellular antiviral activity by exploiting SUMOylation, but indications of a mechanistic link are known. See text for further details on exploitation of the SUMO machinery by single viral proteins. IFN: Interferon; IRF: IFN regulatory factor; PML-NBs: ProMyelocyticLeukemia nuclear bodies.

interferons (IFNs) production by IFN regulatory factor (IRF)-dependent mechanisms[105].

Ebola Zaire virus (EBOV) is a human pathogen that infects initially dendritic cells and macrophages[106], inhibiting the production of the pro-inflammatory IFN type I (IFN- I)[107]. Namely, EBOV VP35 protein potently inhibits IFN- I transcription[108] using the cellular SUMO machinery in dendritic cells. Indeed, VP35 increases SUMOylation of IRF7[109], the principal cellular factor required for IFN- I transcription[110], in a PIAS1 dependent manner. VP35 forms a complex with the SUMO ligase PIAS1 and IRF7, thus increasing PIAS1-mediated IRF7 SUMO-1 and SUMO-3 conjugation[109]. Interestingly, IRFs SUMOylation appears to be a physiological process orchestrating INFs production after viral infection[111], allowing clearing of the infecting virus. Therefore, VP35 exploits SUMO to turn off IFN- I production by dendritic cells, probably worsening the maturation of these cells[112] and weakening the host innate immunity against EBOV infection.

Taken together, these reports strongly suggest the existence of a correlation between SUMO pathway exploitation by viruses and escape from the host innate immune system. However, viruses also possess a large number of mechanisms to escape the intrinsic immune system. This is not surprising, considering that the intrinsic response is the first host defense to fight viral infections.

PML-NBs are nuclear inclusions rich of SUMOylated proteins, known to be crucial organelles involved in intrinsic anti-viral response. In fact, PML-NBs seem to be implicated in the downstream effect of INF-mediated antiviral action[113]. Notably, PML-NBs are disassembled during most viral infections at very early stages, indicating that targeting PML-NBs could be an efficient viral strategy to evade IFN action[113]. Therefore, most of the mechanisms developed by DNA viruses to overcome cellular defense disperse PML-NBs: one rapid way to achieve this goal is by hijacking the SUMOylation pathway.

Herpes simplex virus type-1 (HSV-1) protein ICP0 structure encompasses a RING finger domain that acts as an E3 ubiquitin ligase, redirecting specific cellular proteins for proteasome-dependent degradation[114]. Earlier during infection, PML-NBs components are quickly recruited at sites closely associated with the viral genome in a SUMO-dependent manner[115,116], promoting the transcription of anti-viral genes. However, ICP0 counteracts this PML-NBs response targeting SUMOylated proteins for degradation, thanks to its E3 ligase activity[115]. This HSV-response is strictly required for its infection cycle, since in this way ICP0 inhibits cellular mechanisms that would otherwise repress viral transcription[114]. Interestingly, it has been shown that ICP0 falls in the SUMO targeted ubiquitin ligases (STUbLs) family[117], a class of RING finger ubiquitin ligases that contains SIMs[118]. Therefore, through its SIM motifs, ICP0 binds to important SUMOylated transcription factors in PML-NBs that, in turn, are degraded by the E3 ubiquitin ligase activity of its RING motif. This dual action of ICP0 efficiently counteracts intrinsic antiviral resistance to HSV-1 infection[117].

Like ICP0, KHSV K-Rta protein also belongs to the STUbLs family. Indeed, K-Rta contains SIM motifs and conjugates ubiquitin to SUMO and SUMO-chains, disrupting PML-NBs in a ubiquitin ligase dependent fashion[119].

A similar mechanism is also conducted by Varicella Zoster virus protein ORF61. Indeed, ORF61 colocalizes and disperses PML-NBs shortly after virus entry in its target cell[120]. It also contains three SIM motifs through which it counteracts intrinsic SUMO-promoted anti-viral control by PML-NBs[121]. Consistently, ORF61 SIM mutants are unable to disperse PML and the overall degree of virus infection is dramatically impaired when SUMO-conjugation is inhibited[121]. As for ICP0, PML-NBs dispersal by ORF61 is a two-step process accomplished by different protein domains: the ORF61 SIMs that recognize SUMOylated PML protein in PML-NBs and the RING domain that executes their dispersal[121]. However, ORF61 RING domain does not share the E3 ligase activity with ICP0 and is thus not able to degrade PML-NBs. A similar PML-NBs disruption mechanism seems to be also carried out by the already described EBV BGLF4 protein[91].

While in all the examples described above viruses extensively interact with a number of proteins in PML-NBs, encephalomyocarditis virus (EMCV) counteracts antiviral pathway targeting the PML protein alone[122]. In fact, during infection, PML is first transferred by EMCV from the nucleoplasm to the nuclear matrix and then the viral protease 3C induces PML degradation. Both PML delocalization and degradation are a consequence of covalent SUMO-1, -2 and -3 conjugation promoted by EMCV[122].

In addition to the ability to be itself SUMOylated (see above) for its transactivation functions, CMV IE1 also efficiently inhibits the intrinsic antiviral response by preventing the accumulation of SUMOylated forms of PML[123]. In this regard, CMV seems to behave as EMCV, since IE1 does not induce PML degradation.

CONCLUSION

In recent years, SUMOylation has emerged as a major regulator system involved in a variety of cellular processes. SUMO is indeed conjugated to a number of proteins that in turn can interact with many other partners through the SUMO interacting (SIM) motifs. Therefore, the SUMOylation machinery virtually affects and directs most of cellular activities, crucially regulating cellular homeostasis. Thus, exploiting SUMOylation represents a very convenient way to quickly promote and sustain pathogen survival in the host.

Viruses, in particular, exploit SUMOylation in several key steps of their intracellular life and, importantly, they also use the SUMO pathway to subvert the immune response of the host (Table 1). Both DNA and RNA viruses can use SUMOylation to promote viral genes transcription, virus assembly (Figure 2) and immune evasion (Figure 3), using apparently different mechanisms. Some viral proteins (*i.e.*, E3, E1, L2, A40R, CA, IE1, IE2, BLZF1, Rta, BGLF4, M1, E1B-55k, K-bZip, UL44) are modified by SUMO in order to activate their function; alternatively, they can influence the SUMOylation level of a specific target protein (AL1, VP35) or the global SUMOylation status of infected cells (Gam1, ICP0, K-Rta, L2). Finally, other viral products could interact with SUMO components or with host SUMO-containing proteins (NP, Gag, 3C, ORF61), usually through a SIM motif, or mimicking SUMOylation enzymes (K-bZIP, E1B-55K). Remarkably, the same virus (KSHV, CMV, EBV, Vaccinia Virus, Papillomavirus) can exploit the SUMO pathway through various proteins, as well as the same protein (Gam1, IE1, IE2, E1B-55K, K-bZIP, BGLF4) can interact with SUMO using several mechanisms perhaps also to promote different steps of viral infection.

It is interesting to note that the vast majority of viral proteins known to interact with the SUMOylation system are immediate-early or early proteins, suggesting a crucial role for SUMO in counteracting viral infection.

What we can learn from the complex network of interplay between the SUMO pathway and viruses in the virus-host interactions is that the same crucial pathway can be hijacked by different pathogens in very different ways to obtain a common goal, *i.e.*, sustaining viral infection. More studies are required to define the global picture but the findings presented here can strongly indicate the SUMO pathway as a promising target for specific antiviral therapies.

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TOPIC HIGHLIGHT

Gualtiero Alvisi, PhD, Assistant Professor, *Series Editor*

Effects of human immunodeficiency virus on the erythrocyte and megakaryocyte lineages

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Abstract

Anaemia and thrombocytopenia are haematological disorders that can be detected in many human immunodeficiency virus (HIV)-positive patients during the development of HIV infection. The progressive decline of erythrocytes and platelets plays an important role both in HIV disease progression and in the clinical and therapeutic management of HIV-positive patients. HIV-dependent impairment of the megakaryocyte and erythrocyte lineages is multifactorial and particularly affects survival, proliferation and differentiation of bone marrow (BM) CD34+ haematopoietic progenitor cells, the activity of BM stromal cells and the regulation of cytokine networks. In this review, we analyse the major HIV-related mechanisms that are involved in the genesis and development of the anaemia and thrombocytopenia observed in HIV positive patients.

Key words: Human immunodeficiency virus; Erythrocytes; Megakaryocytes; Haematopoietic progenitor cells; Virology

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INTRODUCTION

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS), which is characterised by the progressive and fatal impairment of immune system function and the occurrence of opportunistic infections and tumours[1]. Although the dysfunction of the immune system and the decline in the number and activity of CD4+ T cells represent the hallmark of HIV infection, it is noteworthy that HIV can also interfere with other cell lineages and tissues[2-5]. In addition to progressive depletion of CD4+ T lymphocytes, peripheral blood cytopenias, such as anaemia, neutropenia and thrombocytopenia, occur in most patients with AIDS[6,7] and in some HIV-positive naive individuals during the early phases of disease progression, especially when high plasma levels of HIV RNA are detectable. Interestingly, isolated thrombocytopenia can represent the first clinical manifestation in otherwise asymptomatic HIV positive patients[8] whereas anaemia and neutropenia are more common in the late stages of

HIV disease[9]. These peripheral blood cytopenias have been observed even in the absence of tumours, chemotherapeutic treatment or opportunistic infections suggesting that HIV infection may be directly associated with the induction of these haematological abnormalities[10]. The progressive depletion of these cell lineages in the blood has been related to several HIV-driven mechanisms: (1) the impairment of survival and proliferation of haematopoietic progenitor cells (HPCs); (2) the inhibition of the differentiation of HPCs into certain cell lineages or direct action on mature cells; (3) the impairment of stromal cells; and (4) the dysregulation of cytokine production and the appearance of autoimmune responses. In this report, we analyse several aspects of these major HIV-related mechanisms that are involved in the impairment of the erythrocyte and megakaryocyte (MK) lineages.

HIV AND CD34+ HPCs

The bone marrow (BM) forms a suitable environment for stem cell survival, growth and differentiation. The cellular components of BM include HPCs, HPC-derived cell lineages and stromal cells. HPCs represent a heterogeneous CD34+ cell population in the BM that includes the most primitive CD34+ haematopoietic stem cells (HSCs), which are characterised by pluripotency and a high capacity for self-renewal, and the CD34+ multi-potent progenitors (MPPs), which originate from HSCs and are multipotent but have a more limited capacity for self-renewal (Figure 1). MPPs can differentiate into common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). CLPs can differentiate into B and T cells, natural killer cells and plasmacytoid dendritic progenitor cells. T cell differentiation occurs in the thymus whereas CMPs differentiate in the BM, through specific differentiation stages, into several cell lineages including granulocytes, erythrocytes, MKs and monocytes[11]. CMP-derived cell lineages migrate into the blood with the exception of MKs, which are maintained in the BM. The differentiation of HSCs is regulated by specific haematopoietic growth factors that induce the survival, proliferation and maturation of specific cell lineages. These factors share several common properties and act hierarchically at different stages of differentiation, and they often show synergistic or additive interactions with other growth factors. Stromal cells are the major source of these factors with the exceptions of erythropoietin (EPO) and thrombopoietin (TPO), which are largely produced in the kidneys and the liver, respectively.

The incidence of peripheral blood cytopenias in HIV positive individuals has led to hypothesis that HIV can impair BM homeostasis and affect the biology and activity of HPCs. Early studies have observed that HIV infection is correlated with the depletion of HPCs and a significant reduction in the *in vitro* growth of HPCs that have been purified from HIV-infected patients[12-17], suggesting that the multiple peripheral cytopenias may be related, at least in part, to a productive HIV infection of

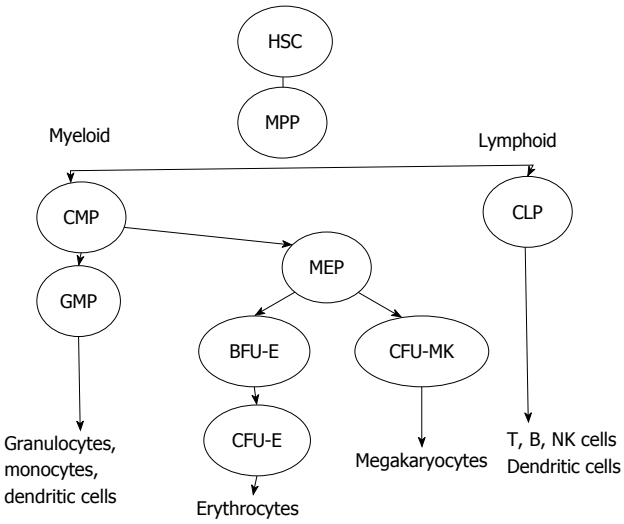

Figure 1 Human haematopoiesis. HSC: Hematopoietic stem cell; MPP: Multipotent progenitor; CLP: Common lymphoid progenitor; CMP: Common myeloid progenitor; GMP: Granulocyte/macrophage progenitor; MEP: Megakaryocyte/erithrocyte progenitor; BFU-E: Burst forming unit-erythroid; CFU-E: Colony forming unit-erythroid; CFU-MK: Colony forming unit-megakaryocyte.

BM HPCs. HIV infection may determine a progressive HPC depletion due to cell lysis, which in turn leads to the derangement of the differentiation towards various cellular lineages. This hypothesis of a potential HIV infection of HPCs may further imply an important feature in the dynamics of HIV disease: long-lived HPCs may harbour proviral HIV DNA genomes in their own genomes and act as an additional reservoir of HIV. Interestingly, cellular HIV receptors and co-receptors can be detected on HPC cell membrane. Flow cytometry analyses showed that 25%-65% of CD34+ HPCs that had been purified from the BM of healthy donors, expressed detectable levels of CD4 protein on their cell membranes[18,19]. Moreover, the CD4 protein was functionally active, and it effectively bound the HIV-1 gp120 anti-receptor[19]. The major co-receptors CXCR4 and CCR5 were also expressed on HPC cell membranes[20-22], and CXCR4 and CCR5 proteins were expressed in 53% and 35% of isolated CD34+ HPCs, respectively[23]. However, the analysis of CXCR4 and CCR5 expression was dependent on the differentiation stage. When the expression levels of CXCR4 and CCR5 were determined in CD34+/CD38- and CD34+/CD38+ HPC subsets, the CXCR4 protein expression level was relatively constant in both subsets whereas CCR5 was detected in 2% of more primitive CD34+/CD38- cells and in 35% of more mature CD34+/CD38+ subset, which indicated that CCR5 but not CXCR4 is up-regulated during differentiation from HSC into MPP[23]. The expression of HIV receptors and co-receptors on the cell membranes of CD34+ HPCs suggested that these cells could be considered a possible target of HIV infection.

To explore this hypothesis, two major experimental approaches were undertaken by several groups: (1) the challenge of BM or cord blood CD34+ HPCs, isolated

from uninfected donors, with HIV strains; and (2) the detection of HIV nucleic acids and/or viral proteins in BM CD34+ HPCs isolated from HIV-positive patients. These studies were based on the isolation and purification of CD34+ HPCs that represent a heterogeneous cell population[24,25] because the CD34+ marker could be detected not only on HSCs and MPPs but also on more committed myeloid progenitors such as CFU-GEMM, CFU-GM, BFU-E and CFU-MK progenitors.

Several reports showed that CD34+ BM HPCs, purified from uninfected donors, were resistant to HIV infection. Polymerase chain reaction (PCR) or reverse transcriptase-PCR analysis of proviral HIV DNA or HIV RNA in HPCs that had been challenged with different HIV-1 strains did not reveal significant evidence of HIV infection[9,12,26-29]. In partial contrast to these data, Chelucci and coworkers[30] have purified CD34+ HPCs from the peripheral blood of healthy donors, cultured them with EPO + granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and SCF and then challenged with different HIV-1 strains. The analysis of p24 protein showed that 12% of CFU-GM and less than 1% of BFU-E colonies were positive whereas the CFU-GEMM progeny were negative.

Interestingly, early stem cells in the CD34+ HPCs, which are arrested in the G_0 phase of the cell cycle, were not permissive for HIV infection[23], and other reports showed that the more primitive CD34+/CD38- HPC subset was not susceptible for HIV-1 or HIV-2 infection[31,32]. However, a limited infection was revealed in the first weeks of long-term culture in CD34+/CD38+ HPCs, which suggested that HIV infects at low extent only the more committed HPC subset but not the more primitive HPCs[31].

The analysis of HIV infection in BM HPCs, purified from HIV-positive patients, was carried out to determine whether these patients could harbour proviral HIV DNA in HPCs. Two studies[33,34], based on PCR assays to detect proviral HIV DNA in BM HPCs, reported that 1 out of 14 patients and 1 out of 11 patients, respectively, were HIV DNA positive. Similar percentages of HIV proviral DNA positive samples were detected in subsequent reports[12,13,35]. In contrast with these results, a higher percentage of HIV-1 infection of CD34+ HPCs was observed in some groups of HIV-1 positive individuals especially in patients with the more advanced stages of the disease[36,37]. This discrepancy could be related to the use of different PCR assays with different sensitivities, and the possible presence of contaminating HIV-infected BM stromal cells.

Notwithstanding these controversial results, the consensus on HPC susceptibility to HIV infection, was that *in vitro* infection of HPCs occurred, under some experimental conditions, in a low fraction of HPCs, and these HPCs were the more committed HPCs, whereas the more primitive HPCs were not considered a significant HIV target. Moreover, *in vivo* infection of HPCs was infrequent suggesting a negligible role of HIV-infection of HPCs in BM derangement and the induction of cytopenias[7].

Several mechanisms have been proposed to explain HPC resistance to HIV infection. HPCs secrete the CCR5 ligands macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and regulated on activation normal T cell expressed and secreted (RANTES)[37,38] and the CXCR4 ligand stromal-derived factor 1 (SDF-1)[22], which may compete with R5- or X4-tropic HIV-1 strain infection by interfering with gp120/co-receptor-binding. In addition, an analysis of the interference between gp120 and mAb directed against CXCR4 in HPCs, suggested the lack of a real CD4/CXCR4 complex on HPC membranes, which excluded the formation of the trimeric complex with gp120, essential for HIV binding and infection[22]. Zhang and coworkers have also showed that the cellular cyclin-dependent kinase inhibitor p21 protein restricts HPC infection and interferes with the integration of the proviral HIV-1 genome[39].

However, recent studies have challenged the consensus about HPC resistance to HIV infection[40-44]. A report has described the HIV-1 subtype C infection in CD34+ HPCs, and the analysis of proviral HIV DNA in peripheral blood CD34+ cells showed that 12 out of 19 patients were positive. Interestingly, HIV-1 subtype B strains were not able to infect HPCs, suggesting that only specific HIV subtypes could be associated with direct infection of HPCs[40]. Carter *et al*[41] challenged purified HPCs with a molecular HIV clone p89.6 derived from the dual tropic HIV strain 89.6. A small percentage (1%-6%) of HPCs exhibited HIV-1 gag protein expression 72 h post infection. A similar infection rate was found, in contrast with previous studies, even in the more primitive CD133+CD34+CD38- HPC subset. However, the methodological approach of this study was subsequently criticised for the choice of the sole criterion of gag analysis, the infection protocol and the pseudo-viruses that were used[45]. These results were substantially confirmed by the same group in a subsequent study[42] that showed HIV infection in approximately 2% of primitive CD133+CD34+^{high} HPC subset cells. HIV infection was detectable when X4-tropic HIV subtype B strains were used, whereas R5-tropic HIV strains were ineffective on CD133+CD34+CD38- HPC subset cells, suggesting that the infection of HPCs might be detectable when X4-tropic HIV strains appear during the progression of HIV infection. These X4-tropic HIV strains are generally observed in the late stages of HIV infection and are related to more rapid disease progression and a poorer prognosis.

Carter *et al*[41] have also studied BM HPCs, isolated from six HIV-positive patients with high HIV RNA load. HIV-1 gag protein was detected in three of the six samples. When these cells were cultured with GM-CSF and tumour necrosis factor-α (TNF-α) to induce myeloid differentiation, all six of the samples were positive for the gag protein. In the same report, fresh BM HPCs, isolated from nine combination antiretroviral therapy (cART)-treated HIV positive individuals with undetectable viral loads for longer than 6 mo, were analysed using a quan-

titative real time PCR assay for integrated proviral HIV DNA. Four of the nine samples were positive with the number of proviral HIV genomes ranging between 2.5-40 copies/10000 CD34+ HPCs. These data suggested a new interpretation of the interaction between HIV and HPCs, in which a low number of HSCs and HPCs are susceptible to HIV infection and may represent an HIV reservoir. The rate of infection could be under-recorded because the data from Carter *et al*[41] indicate that HIV challenge is cytotoxic for HPCs. Moreover, their studies showed that even HIV-1 subtype B strains could infect these HPC subsets, which is in contrast to the previous study indicated above[40], suggesting that the HIV-1 subtype B cytotoxicity could explain this phenomenon. The infection of HPCs indicated that these cells could be a reservoir of HIV. Unfortunately, the hypothesis of HPCs as a viral reservoir was not confirmed by two subsequent analyses on proviral HIV-1 DNA in HPCs[46,47]. In these studies, CD34+ HPCs were purified from 11 and 8 HIV-positive patients treated with long-term suppressive cART. High sensitivity PCR assays demonstrated no HIV-1 proviral DNA in these cells[46,47].

Altogether, these recent studies have reconsidered the relationship between HIV infection and HPCs, but the data still remain controversial and further studies are needed to evaluate whether HIV infection of HPCs may be associated with the onset of blood cytopenias or may represent an additional HIV infection reservoir.

In addition to analyses of the direct infection of HPCs by HIV, several studies have been performed on granulocyte-macrophage CFUs (CFU-GMs), mixed lineage CFUs (CFU-GEMMs) or erythroid burst-forming units (BFU-Es). In this context, HPCs from HIV-1 infected patients, showed impaired *in vitro* BFU-E, CFU-GM and BFU-MK growth[12,25,27,34,36,48,49]. These results were confirmed even in HPCs purified from HIV-negative individuals and challenged with HIV even though other studies did not observe growth inhibition[26,30,50,51] probably due to the different HIV strains and cell culture conditions that were used. The significant reduction of CFU-GEMM, CFU-GM, BFU-E, and BFU-MK growth suggested an alteration of HPC proliferation with the possible involvement of apoptosis in the induction of cytopenias. Apoptosis plays an important role in the depletion of CD4+ T lymphocytes even through the interaction of HIV gp120 and CD4. HIV gp120 is also able to induce the activation of apoptosis in endothelial cells, osteoblasts, and neurons[52-54], and several studies have been performed on the effects of heat-inactivated HIV and certain viral proteins such as gp120 and Tat on the myelosuppression observed in HIV-positive patients. HIV-1 gp120 and heat-inactivated HIV-1[28,31,55,56] impaired the *in vitro* clonogenic capacity and induced apoptosis. This negative regulation of proliferation and survival was associated with transforming growth factor β1 (TGFβ1) increased production by HPCs and the occurrence of a Fas-dependent mechanism[57,58]. This reduction in survival and proliferation due to apoptosis could at least partially explain the decrease of HPCs and circulating precursors that has been noted in HIV-positive patients[13,34,36,56-62].

HIV AND BM STROMAL CELLS

The cellular components of the BM include HPCs at all stages of differentiation and stromal cells. BM stromal cells are a mixed population composed of mesenchymal stem cells (MSCs), endothelial cells, macrophages, fibroblasts, adipocytes, osteoblasts and osteoclasts, as well as dendritic cells and B and T lymphocytes that migrate from the blood to the BM. Stromal cells are essential for proper homeostasis and the regulation of BM haematopoiesis through a complex cellular cross-talk that is modulated by cytokines. *In vitro* experiments using long-term BM cultures showed that HIV-infected BM stroma was unable to support uninfected CD34+ HPC growth and differentiation compared to uninfected cultures[49,63,64]. In addition, the stromal structure of the BM in HIV patients shows morphological variations including an increased number of macrophages and a decreased number of fibroblasts[10,65]. This impairment of stromal activity and structure affects HPC differentiation and growth and it is due to the complex interaction between HIV and the different BM stromal cells that lead to a derangement of cytokine regulation. In particular, certain cell types, such as T cells, MSCs, macrophages and endothelial cells, are targeted, directly and indirectly, by HIV and its proteins including Tat and gp120[66-69]. BM MSCs can differentiate towards several cell lineages such as osteoblasts, adipocytes, fibroblasts, *etc*. *In vitro* experiments have demonstrated that HIV, gp120 and Tat can elicit a derangement of the clonogenic capacity of BM MSCs. In particular, the osteoblast differentiation is inhibited whereas adipocyte differentiation is increased. The alteration of the clonogenic activity may also explain the decreased number of fibroblasts that are detectable in the BM of HIV patients[10,65]. T cells, macrophages, endothelial cells and MKs are productively infected by HIV to different degrees in the BM. Endothelial cells are permissive for HIV infection and BM endothelial cells are infected in HIV patients at every stage of HIV disease. Endothelial cell infection was related to BM impairment in HIV-positive subjects because they exhibited a reduced ability to respond to BM micro-environmental regulatory signals that positively up-regulated the number of blood cells[69].

CD4+ T cells and macrophages are the major targets of HIV replication, and the release of specific cytokines and haematopoietic factors is affected by HIV infection. TNFα, TGFβ1, interferon-γ (IFN-γ), IL-1, IL-6, IL-10, IL-18, TNF-related apoptosis-inducing ligand and monocyte colony-stimulating factor are dysregulated by HIV in T cells and monocyte models[70-75]. Similarly, viral proteins such as Tat and/or gp120 increase the expression of IL-6, TNFα and IL-1[76-81]. The impairment of several cytokines during HIV infection was confirmed by clinical studies in which higher levels of IL-1, IL-18, TNFα and IL-6 in the plasma of HIV-positive patients were

detected compared to uninfected individuals[82-85]. It is noteworthy that the pro-inflammatory cytokines TNFα, IL-1, and IL-6 and the chemokines MIP-1α, MIP-1β and RANTES were up-regulated in the BM of HIV-positive patients[65,86]. TNFα involvement in the HIV-1-induced suppression of haematopoiesis, was also suggested in neutralisation studies[87]. Tat is able to elicit a significant activation of the TGFβ1 expression in macrophages that have been isolated from BM. BM macrophage culture supernatants were added to BM HPC cultures thus inducing an inhibition of HPC growth in the liquid cultures[88]. This chronic derangement of cytokine modulation can elicit several negative effects on HPCs and their differentiation into various cell lineages, cooperating in the pathogenesis of anaemia and thrombocytopenia in HIV-infected patients.

HIV AND THE MK LINEAGE

Chronic thrombocytopenia is detectable during HIV disease in approximately 10% of HIV positive patients and 15%-60% of patients with AIDS[89-92]. This haematological disorder may represent the first manifestation of HIV infection and it may progress over time and lead to severe bleeding[91]. HIV-associated thrombocytopenia is related to reduced platelet survival, ineffective platelet production and the impairment of the survival of BM MKs and their precursors. HIV targets the MK cell lineage by interfering throughout the differentiation of mature MKs (Figure 2A). As described above, HIV decreases the number and activity of HPCs and induces a growth deficit in CFU-MKs in HIV patients. An analysis of the impact of HIV-1 and gp120 during TPO-induced cord blood-derived HPC differentiation into MKs has demonstrated that gp120 treatment led to the induction of apoptosis in the CD41+ and CD61+ subsets due to TGFβ1 increase and APRIL down-regulation[55]. These data confirmed the induction of apoptosis through the gp120 engagement of CD4, observed in BM GP Ⅱb/Ⅱa+ (CD41+) megakaryocytic cells and in megakaryocytic cell line models[93-95]. Moreover, a reduction of c-mpl expression in the MK lineage due to V3 loop region of gp120 was observed in MK lineage thus indicating a further mechanism involved in the impairment of megakaryocytopoiesis[96].

HIV and gp120 altered the maturation of MKs, and decreased the number of MKs with higher ploidy[55]. Electron microscopy analysis of MKs from HIV-infected individuals with thrombocytopenia clearly demonstrated ultrastructural abnormalities, such as blebbing of the surface membrane and vacuolisation of the peripheral cytoplasm[97]. Mature MKs can be infected by HIV through binding the CD4 receptor[97-102], and HIV genomes have been detected in MKs purified from BM of HIV-positive patients[103]. The infection of MKs is not strain-restricted because both R5- and X4-tropic HIV-1 strains are able to infect MKs thus indicating that the infection may occur early in the development of HIV infection[99]. In addition to these direct effects of HIV on the MK cell lineage,

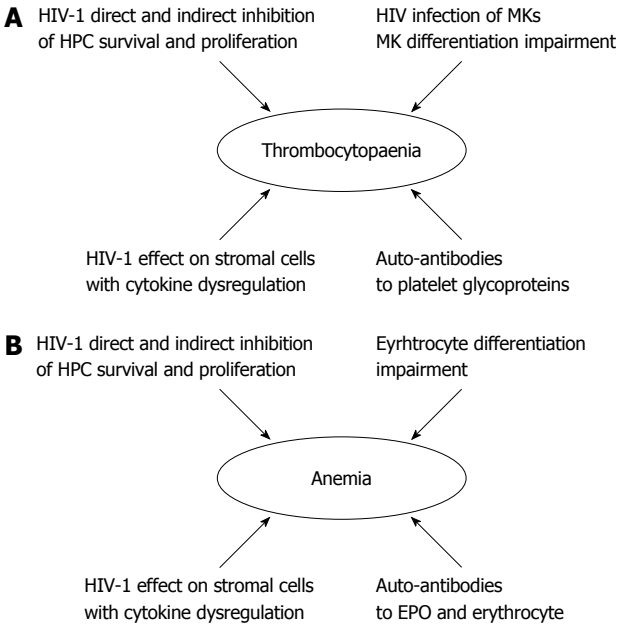

Figure 2 The mechanisms involved in human immunodeficiency virus-related thrombocytopenia and anaemia. A: Human immunodeficiency virus (HIV)-related thrombocytopenia; B: HIV-related anaemia. EPO: Erythropoietin; HPC: Haematopoietic progenitor cell; MK: Megakaryocyte.

HIV also supports chronic thrombocytopenia through autoimmune mechanisms[89-92], particularly evident in early stages of the disease[104,105]. Autoimmune mechanisms are related to anti-HIV antibodies cross-reacting with platelet-membrane glycoproteins, supporting the basic role of molecular mimicry in the induction of these antibodies[106-110]. In particular, an autoantibody directed against integrin GP Ⅲa49-66 induced a platelet lysis[110] and cross-reacted with some peptides derived from Nef and gp120[111]. The anti-GPⅢa49-66 antibody isolated from HIV-1 patients down-regulated MK proliferation in *in vitro* culture of human cord blood CD34+ cells driven by TPO[112].

Platelets can bind HIV-1 gp120 through its CXCR4 and fibronectin surface receptors, and platelet-bound HIV may infect permissive cells suggesting a possible role for platelets as carriers in the spread of HIV infection[113]. The interaction between platelets and HIV leads to the activation of platelets and an altered platelet morphology, which is likely due to CXCR4 binding because this protein is the receptor of SDF-1, a factor involved in enhancing platelet activation by agonists[114]. Platelet activation was detected in HIV patients and the degree of activation in circulating platelets was higher in AIDS patients than patients in earlier stages of HIV infection[115]. Activated platelets also represent a source of some pro-inflammatory cytokines. Their activation led to a strong induction of IL-1β and IL-18 secretion eliciting a further cytokine regulation derangement[114,116]. These alterations of platelet activity were also related to the impairment of coagulation homeostasis, thus increasing the complexity of the HIV/MK/platelet/coagulation interactions. These studies demonstrated that the MK lineage is a direct and

indirect target of HIV and its proteins throughout their entire differentiation and development. This targeting affects platelet maturation and activity, explaining why thrombocytopenia is a major cytopenia in HIV-positive patients.

HIV AND THE ERYTHROCYTE CELL LINEAGE

Anaemia is a clinical complication detectable in many HIV patients[117]. The overall incidence of anaemia in HIV-positive individuals is 10% in asymptomatic patients and up to 92% in patients with AIDS[6,117]. cART treatment has reduced but not solved the problem of anaemia in HIV patients. In a cohort of 1624 patients in the EuroSIDA study, the prevalence of anaemia during HAART decreased from 65% in naive patients to 53% after 6 mo of therapy and 45% after 1 year of therapy[118]. Although anaemia does not generally cause death in HIV patients, it is well known that anaemia can increase morbidity in these subjects. HIV patients with anaemia have a higher risk of reduced survival compared to non-anaemic in HIV positive patients[117,119]. The symptoms of anaemia during HIV infection are not different from the symptoms that are observed in HIV negative patients, and the diagnosis of anaemia is often a laboratory diagnosis based on a reduction of the haemoglobin (Hb) value and erythrocyte count. The anaemia is generally mild with Hb concentrations between 8-14 g/dL for men and 8-12 g/dL for women, although the degree of anaemia is dependent on the immunosuppressive context and disease stage[120]. The erythrocyte morphology does not exhibit consistent variations in the peripheral blood[121]. Microcytosis is rarely observed, whereas macrocytosis is found in HIV-positive patients treated with zidovudine (AZT). AZT treatment was related to BM suppression, and the HER and WIHS studies observed a significant increase in anaemia in AZT-treated patients[122,123]. Some reports indicated the presence of poikilocytosis, anisocytosis and ruleaux formation, but, in general, HIV-associated anaemia is characterised by normocytosis, low reticulocyte counts and an ineffective erythropoiesis with an hyporegenerative BM[6,124]. The pathogenesis of anaemia in HIV-positive patients is multifactorial (Figure 2B): the different mechanisms that are involved in the anaemia induction are characterised by the impairment of erythrocyte production and increased erythrocyte destruction[120]. HIV is directly involved in the induction of anaemia even though neoplastic diseases, vitamin deficiencies, iron metabolism impairment, pharmacological treatments and opportunistic infections are implicated in anaemia onset during HIV infection. The involvement of HPCs in the cytopenias has been illustrated above, however, it is noteworthy that Cleveland and coworkers observed the expression of CD4 on the cell membranes of erythroid differentiating cells. The co-expression of CD4 and glycophorin A indicates that some erythroid-committed cells could represent a target for HIV infection[125]. In addition, the expression of functional CXCR4[37] was detected in CD34+ BFU-Es even though its expression level decreased during erythroid differentiation. Interestingly, Tat treatment of cord blood-isolated HPCs up-regulated CXCR4 protein expression indicating a complex effect of HIV activity on erythrocyte lineage survival and differentiation[126]. Moreover, the dysfunction of erythroid differentiation could be related to BM microenvironment damage and stromal cell impairment[7,71]. IL-1β, IFN-γ, TGFβ1 and TNFα suppress the growth of progenitor cells *in vitro* and may play an important role in the induction of HIV-associated anaemia[116,126,127]. Some papers have suggested that HIV could impair the EPO-related feedback mechanisms that regulate the red blood cell homeostasis. Decreasing the Hb concentration induces EPO production, whereas in many HIV patients the presence of anaemia is coupled with a decrease in the serum EPO concentration that is independent of kidney damage[121,128,129]. Moreover, *in vitro* experiments demonstrated that HIV-1 reduced EPO synthesis[130]. Different mechanisms have been considered to explain this EPO reduction. HIV-related up-regulation of pro-inflammatory cytokines IL-1β and TNF-α directly down-regulates EPO expression *in vitro*[131] through the cytokine-mediated formation of reactive oxygen species, which in turn impair the binding affinities of EPO-inducing transcription factors. In addition, circulating antibodies to EPO are detectable in approximatively 23% of HIV-infected patients, and a prospective study on 113 patients showed that anti-EPO antibodies could be considered an independent risk factor for anaemia[132,133]. The presence of these auto-antibodies, directed against several targets, was associated with molecular mimicry and the dysregulation of the immune system. Recent reports demonstrated that the anti-EPO antibodies recognised three major EPO molecule epitopes that span three regions including the amino acids domains 1-20 (EP1), 54-72 (EP5) and 147-166 (EP12) of which EP1 and EP12 are the domains that are involved in the EPO-EPOR interaction[134]. The region corresponding to EP1 shows a 63% sequence homology with the 34-52 amino acid sequence of HIV gag p17, and a cross-reaction between anti-EP-1 auto-antibodies and the gag fragment was detected suggesting a role for mimicry by this protein in the occurrence of anaemia[134]. HIV-associated anaemia could also be induced by haemolysis. In HIV patients, cases of haemolysis have been observed that are linked to CID, glucose-6-dehydrogenase deficiency, auto-antibodies against red blood cells, thrombotic thrombocytopenia purpura and pharmacological treatment. Furthermore, some HIV positive patients exhibited the presence of a broad panel of specific and non-specific anti-erythrocyte antibodies, and, in some cases, erythrocyte lysis was mediated by complement activation. Although consistent haemolysis is rare in HIV patients, the damage and lysis of red blood cells by auto-antibodies can be considered an additional mechanism of HIV-associated anaemia[135-137].

In conclusion, the occurrence of thrombocytopenia and anaemia represent major pathological manifestations

in HIV patients. The pathogenesis of these cytopenias is multifactorial, and several targets such as HPCs, cell lineage differentiation, cytokine dysregulation and stromal cell impairment cooperate in the occurrence of these haematopoietic defects. The investigation of the different mechanisms that are involved in the genesis of these cytopenias has provided important findings on HIV pathogenesis even though some pivotal items such as the susceptibility of HPCs to HIV infection and their role as HIV infection reservoirs are still under debate and deserve additional experimental analysis. Further studies will be essential to better characterise these mechanisms and to identify useful targets for supportive therapy and management of HIV-positive patients.

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TOPIC HIGHLIGHT

Gualtiero Alvisi, PhD, Assistant Professor, *Series Editor*

How virus persistence can initiate the tumorigenesis process

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Abstract

Human oncogenic viruses are defined as necessary but not sufficient to initiate cancer. Experimental evidence suggests that the oncogenic potential of a virus is effective in cells that have already accumulated a number of genetic mutations leading to cell cycle deregulation. Current models for viral driven oncogenesis cannot explain why tumor development in carriers of tumorigenic viruses is a very rare event, occurring decades after virus infection. Considering that viruses are mutagenic agents *per se* and human oncogenic viruses additionally establish latent and persistent infections, we attempt here to provide a general mechanism of tumor initiation both for RNA and DNA viruses, suggesting viruses could be both necessary and sufficient in triggering human tumorigenesis initiation. Upon reviewing emerging evidence on the ability of viruses to induce DNA damage while subverting the DNA damage response and inducing epigenetic disturbance in the infected cell, we hypothesize a general, albeit inefficient hit and rest mechanism by which viruses may produce a limited reservoir of cells harboring permanent damage that would be initiated when the virus first hits the cell, before latency is established. Cells surviving virus generated damage would consequently become more sensitive to further damage mediated by the otherwise insufficient transforming activity of virus products expressed in latency, or upon episodic reactivations (viral persistence). Cells with a combination of genetic and epigenetic damage leading to a cancerous phenotype would emerge very rarely, as the probability of such an occurrence would be dependent on severity and frequency of consecutive hit and rest cycles due to viral reinfections and reactivations.

Key words: Virus; Carcinogenesis; Tumor; Oncogene; Latency; Viral persistence

Core tip: Current models for viral driven oncogenesis cannot explain why tumor development in carriers of tumorigenic viruses is a very rare event, occurring decades after virus infection. Considering that viruses are mutagenic agents *per se* and human oncogenic viruses additionally establish latent and persistent infections, we attempt here to provide a general mechanism of tumor initiation both for RNA and DNA viruses, suggesting viruses could be both necessary and sufficient in triggering human tumorigenesis initiation.

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TUMORS AND VIRUSES

According to currently accepted estimates, viruses are etiologically linked to 15%-20% of all cancer cases worldwide[1-3]. Although many animal and human viruses can transform cells upon infection, only six human viruses are consistently associated with the onset of tumors in man,

Table 1 Oncogenic viruses are latent/persistent viruses

Virus	EBV	HHV-8	HPV	HBV	HCV	HTLV-1
Associated tumor(s): viral protein(s) expressed	BL: EBNA-1 NPC, TCL: EBNA1 + LMP1 HL: EBNA1 + LMP1-2 PTLD: EBNA1-6 + LMP1-2	KS: vFLIP, vCYC, LANA-1 PEL, MCD: vFLIP, vCYC, LANA-1, LANA-2, vIL-6	Anogenital, oral, skin and laryngeal cancers: E6, E7	HCC: HBx	HCC: CP10, NS3, NS5	ATL: tax
Persistency	Always	Always	20% infected subjects	90%-95% infected newborns 5% infected adults	70%-85% infected subjects	Always
Period between infection and tumor onset	10-20 yr	10-20 yr	5-20 yr	10-30 yr	10-30 yr	20-30 yr

EBV: Epstein-Barr virus; HHV-8: Human herpesvirus 8, also named Kaposi sarcoma virus; HPV: Human papillomavirus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HTLV-1: Human T-cell leukemia virus 1; BL: Burkitt lymphoma; NPC: Nasopharyngeal carcinoma; TCL: T cell lymphoma; HL: Hodgkin lymphoma; PTLD: Posttransplant lymphoproliferative disorder; KS: Kaposi sarcoma; PEL: Primary effusion lymphoma; MCD: Multicentric Castleman's disease; HCC: Hepatocellular carcinoma; ATL: Adult T-cell leukemia.

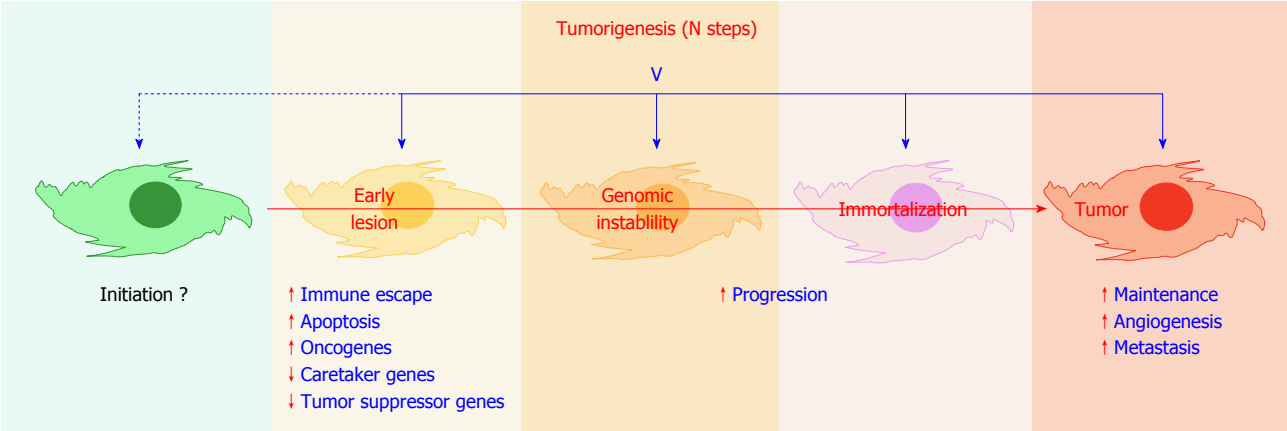

Figure 1 Viral infection and tumorigenesis. Viruses have been shown to encode functions that can modulate all crucial steps towards tumor development, with the exception of the initiation step(s). Recognized contributions of viral infection are mentioned in blue letters. V: Virus. Red arrowheads up, stimulation; down, inhibition.

namely human papillomavirus (HPV), human T-cell lymphotropic virus 1 (HTLV-1), Epstein-Barr virus (EBV), human herpesvirus 8 (HHV-8), hepatitis B virus (HBV) and hepatitis C virus (HCV) (Table 1). A large share of what is known about the molecular mechanisms of oncogenesis is due to studies of tumor viruses, defined thereof as viruses carrying in their genome one copy of an oncogene or of an anti-oncogene or viruses that can alter the expression of the cellular version of one such gene[4]. Viruses have been shown to influence tumor sustainment and progression and induce escape pathways from apoptosis and immune surveillance[1,4], however in no case has it been proven that a virus can be the initiator, the primum movens, and not merely an "influential passenger" of a tumor (Figure 1).

TUMORS AND GENES

Tumor development is believed to be a multistep process leading to the accumulation of permanent genetic damage[5], affecting either oncogenes, tumor suppressor genes, or stability genes[6,7]. Cancer is therefore essentially a genetic disease, and a crucial observation in understanding multistep carcinogenesis is that the vast number and the coarse/crude nature of chromosomal defects that are present in the majority of tumor cells[8], are not amenable to an altered mutation rate in these cells[9,10]. In fact, most human solid tumors are characterized by an abnormal chromosome content, aneuploidy, which can be caused by genetic instability[8,11,12]. In addition, distinct and inheritable gene expression and phenotypic states that arise independently from changes in DNA sequence, known as epigenetic modifications, are also linked to tumor formation and progression[13,14]. On the whole, mechanisms for the initiation of tumorigenesis leading to genetic instability are on the whole poorly understood, both for virus induced and virus unrelated tumors[6].

CAN VIRUSES INITIATE GENETIC INSTABILITY?

It has been known for more than four decades that

Table 2 Viral proteins inducing genetic damage

Virus	EBV	HHV-8	HPV	HBV	HCV	HTLV-1
Latent proteins	EBNA-1 EBNA-3C LMP-1[63]	LANA-1[64] v-CYC[65]	E6, E7[66,67]	Naturally occurring pre-S mutants[68]	-	-
Lytic proteins	BZLF-1[69] BGLF-5[70]	-	E1, E2[71,72]	HBx[73,74]	Core NS3[23,62] NS5[75]	Tax[76,77]

EBV: Epstein-Barr virus; HHV-8: Human herpesvirus 8, also named Kaposi sarcoma virus; HPV: Human papillomavirus; HBV: Hepatatis B virus; HCV: Hepatitis C virus; HTLV-1: Human T-cell leukemia virus 1.

Table 3 Virus products controlling cellular epigenetic modifications

Virus	EBV	HHV-8	HPV	HBV	HCV	HTLV-1
	EBNA-3A, EBNA-3C[78] LMP-1[79] LMP-2[80]	LANA-1[81] microRNA[82]	E6[83] E7[84]	HBx[85,86]	Core[87]	Tax[88]

EBV: Epstein-Barr virus; HHV-8: Human herpesvirus 8, also named Kaposi sarcoma virus; HPV: Human papillomavirus; HBV: Hepatatis B virus; HCV: Hepatitis C virus; HTLV-1: Human T-cell leukemia virus 1.

members of different virus families can induce chromosome damage in infected cells *in vitro*[15], and chromosome breakages have been observed in leukocytes isolated from patients experiencing systemic viral active infections[16,17]. In recent years evidence has accumulated indicating the ability of different viruses to induce aberrant mitosis, genetic instability and interfere with cellular DNA repair pathways, which has confirmed early reports[18-21]. Recent data suggest that viruses induce permanent damage in the genome of infected cells in the context of their natural infection[22,23], and are capable of chromatin manipulation and epigenetic reprogramming of host expression patterns[24,25]; it remains to be seen whether this could stimulate tumor initiation.

It is well known that viruses can transform non-permissive cells and several human viruses cause tumors if introduced in experimental animals. Interestingly all of the six human oncogenic viruses are able to establish latent and persistent infections (Table 1). Chronic HBV, HCV and EBV infections, persistent infection with HTLV-1, prolonged exposures or frequent reactivations of HPV and HHV-8 associated with clinical conditions, are all epidemiologically linked to increased risk of developing virus related malignancies[26-32]. Failure to eliminate emerging tumor cells because of impaired immune function alone cannot account for this increased risk, since tumors develop in a minority of immune depressed patients. Furthermore tumor cells emerge very rarely from *in vitro* virally transformed cell lines, growing in the absence of immune selective pressure[33]. When they do, these tumors are not associated with genetic instability[34]. Therefore there is a missing causative factor acting in the setting of persistent infections, generally thought of as non viral carcinogens or host responses[35]. We propose that reiteration and severity of infections/reactivations is a key factor that possibly generates primary genetic and epigenetic damage on which viral oncogenes may add up their own oncogenic activities.

A MECHANISM FOR TUMOR INITIATION IN VIRAL PERSISTENCE

Viral persistence can be achieved by continuous replication, latency or both. Several virus-encoded products have been associated with transforming and/or oncomodulatory activities[4], and with the ability to induce chromosome damage, abnormal mitosis and genetic instability when expressed in cell cultures (Table 2)[18,36-38]. Recent findings point to viral proteins interfering with the epigenetic milieu of the infected cells, leading to the transcriptional repression of tumor suppressor genes, and interference with cell cycling control[39] (Table 3). However *in vivo* these activities must be particularly inefficient if one considers that the majority of the human population carries a number of resident viruses, but only a minority among infected individuals will develop tumors that can be correlated with persistent viral infections, and generally after very long latency periods (years to decades)[4,35]. It should be noted that latency is characterized by a relatively low viral transcriptional rate[40,41], that one can define as "a virus at rest": this could explain why damaging and/or destabilizing activities of latent gene products have little chance to induce permanent effects in cells equipped with an intact set of caretaker genes, antioncogenes, and non activated oncogenes. Consistently, subjects with Fanconi's anemia, an inherited disease with defective DNA repair, have up to 4000 times increased risk of developing solid papillomavirus-associated tumors[4]. In fact cell immortalization has been achieved experimentally only following expression of latent genes in the context of previously accumulated mutations in the cellular genome[18,20]. On the other hand, lytically infected cells are typically characterized by massive transcription of the viral genome, a "hit". These cells develop virus induced chromosome damage and can undergo abnormal mitosis (Table 2), both *in vitro* and

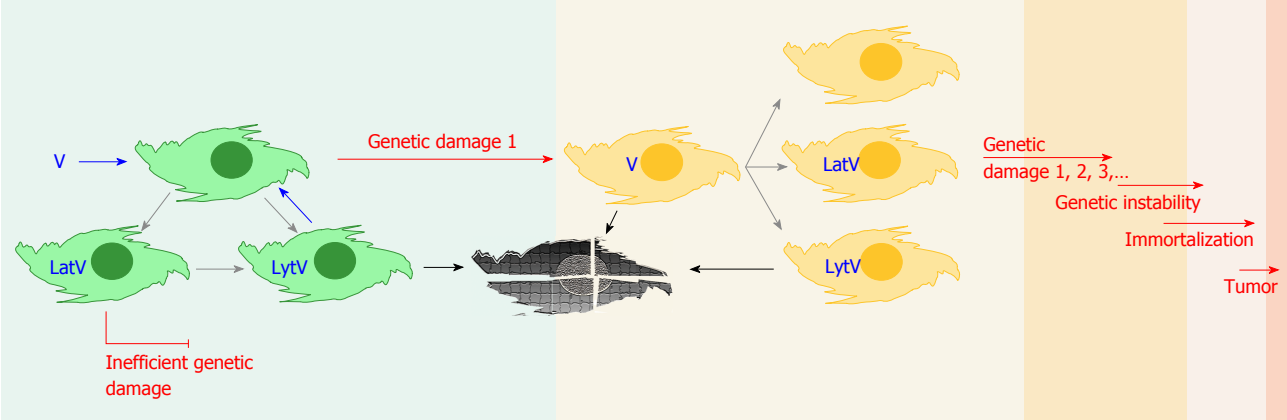

Figure 2 Tumor initiation events mediated by virus induced genetic damage. Virus entry into permissive genetically intact cells (green cells), can result in lytic replication or latency. In the latter setting the oncogenic potential of latent genes appears ineffective *in vivo*. Before silencing of most virus specific transcription is achieved, various viral functions are expressed which could induce genetic/epigenetic damage in a fraction of the infected population (red arrows, genetic damage 1). Cells surviving to sustainable damage (orange cells) could experience reactivation of the virus, host the virus genome in a latent state, or lose it after uneven segregation of their genetic material. In damaged cells latent gene products could now represent an effective oncogenic threat if cellular caretaker genes have been affected (red arrow, genetic damage 1, 2, 3…). Reinfection or reactivation of latent virus in damaged cells could result in further genetic offense, eventually leading to genetic instability, immortalization and tumor development. V: Virus; Lat: Latent; Lyt: Lytic. Blue arrows: Infection; Grey arrows: Consequences of infections; Black arrows: Death.

in vivo[16]. So here we have two observations where there is apparently little if any effect on the genetic stability of healthy cells *in vivo*: (1) latency functions can transform cells but cause genetic instability only in already genetically damaged cells; and (2) lytic functions may induce genetic instability but kill the cells. Is there a setting in which these two phenomena may lead to an outcome that has been overlooked? The phase immediately following virus entry into a permissive cell, before the fate of the infection (lytic or latent) is set (green cells in Figure 2), may be crucial in this regard.

Latency is defined as an infection where the production of infectious virus does not occur immediately but the virus retains the potential to initiate productive infection at a later time, and is characterized by a unique transcriptional and translational state of the virus, the latency expression program, in which the productive replication cycle is not operative[4]. Hence latency can be regarded as a transitory state of resistance of the infected cell to a virus, and the latency program as the result of a negotiation between virus and host, after a battle between cellular functions reacting to the incoming virus and virus encoded functions, expressed at early stages following virus entry into the host cell. The first consequence of this definition is that latency is not a default life program for a virus, but a survival condition that a virus is forced to opt for when the infected cell does not allow progression of the lytic cycle. A second consequence is that there is not one strictly planned latency program for any given virus, but the latency program is defined depending on the context of host cell gene expression, after the cell succeeds converting a viral hit into a virus at rest by resisting to the initial round of lytic cycle gene transcription, and forces the virus into the latency state, for some time. This state of resistance can last for very different periods of time, depending on the moment viral reactivation will be allowed by the infected cell, and can be long lasting, when reactivation occurs upon transition into a new cell differentiation state, or last an unpredictable period of time, as in cells entering a particular metabolic state triggered by an infrequent external signal (ultraviolet radiations, stress, *etc.*), or cells being in a particular phase of the cell cycle[42], which could be a frequent event for rapidly replicating cells or a very infrequent event for slowly replicating cells, as for liver cells. Recent evidence reveals that in an EBV latency model lytic genes can be transcribed to considerable levels[43], contrary to what had been thought previously. Similarly in Kaposi sarcoma virus associated tumors, subpopulations of cells express lytic gene products within a general latency setting[44], suggesting the distinction between latency and lytic transcription is less clear cut than expected. But what happens between viral entry into a cell and the establishment of latency in that cell? Very few studies have addressed this issue, but available data indicate that during this time lapse the majority of the viral genome is transcriptionally active, with many lytic genes being expressed in very much the same way as during early phases of lytic infection, before transcriptions are silenced by the host cell[45,46]. This delicate, vastly unexplored resistant period may represent a particularly vulnerable setting for the infected host, acting as a non-permissive cell, a well known target for virus transformation[1,47]. Therefore the actual phase between virus entry and the establishment of latency is a stage where some viral genes, whether belonging to the latent or the lytic expression program, can be expressed to various levels. Additionally, this is a time where the structure of the incoming virus disaggregates within the cell, releasing dozens of structural proteins and enzymes, genomic nucleic acids, coding and non coding RNAs, encapsidated in infectious particles. In fact it is now clear that the presence of incoming viral genomes relocates DNA repair proteins at sites of viral genome deposition[48]. Several virus products are able to induce genetic damage (Table 2), and examples

of encapsidated DNA nicking activities with a potential role in chromosomal damage have been reported[49,50]. Other viral proteins can interfere with the cellular DNA repair machinery (see[51] for a detailed review) or introduce transcriptional-silencing marks[39]. All these activities could in this context generate primary damage events, leaving the cell with permanent genetic and epigenetic damage before entering into latency. The ensuing latency program would now run in a cell bearing a modified genome.

Although it would be reasonable to expect that the majority of damaged cells could not survive the insult, it would be equally reasonable to expect that cells with sustainable damage may survive, as it is documented *in vitro* in non-permissive cells[19,52] and in cells undergoing chemically induced DNA breaks and chromosome pulverization[53].

A surviving cell could be imagined as acquiring a genotype with no phenotypic consequences on the virus, in which case the virus would either proceed with the lytic cycle and kill the cell or enter a latent state (rest), according to the virus and the type of infected cell (Figure 2). Alternatively genetic/epigenetic damage could modify the permissivity of the cell to the infecting virus, either further supporting viral expression programs or restricting them. The consequences on lytic infections would be either more productive lytic cycles or their inhibition with possible elimination of the virus, respectively. On latent infections the expression profile of the genome could be affected, either positively, as observed in EBV positive NK/T-cell lymphoma[54], or negatively as it is observed when EBV latently infected B cells switch from the latency Ⅲ (whole set of latency products expressed) to latency I (EBNA-1 only) following transformation into lymphoblastoid cells. Viral gene expression would now take place in the context of a genetically modified cell, and in some instances this combination could provide damaged cells with a selective advantage in their environment, making them fitter to survive such damage and ready for the accumulation of future genetic modifications, in other words placing them on the road to malignancy.

IS THE VIRUS LATENCY/REACTIVATION CYCLE AN ONCOGENIC THREAT?

While a single hit and rest event has little chance to set the stage for cancer initiation, repeated cycles of viral infection or reactivation and latency would increase the number of possibly genetically damaged cells in the host and eventually produce cells accumulating a number of chromosomal abnormalities, as recently observed in an *in vitro* model by Fang *et al*[55]. If the damage has modified or abolished the activity of caretaker genes, oncogenes or anti-oncogenes, then the genome damaging and/or destabilizing activities of viral latent gene products could now meet the requirements for the introduction of additional permanent damage, eventually leading to genetic instability. When the combination of hit and rest related damage reached a critical point, let's say telomerase activation, the cell could become immortal and virus functions may become dispensable. Further damage due to genetic instability could lead finally to the emergence of a tumor cell (Figure 2). If the present hypothesis was confirmed, one consequence would be that the number of viruses with potential for tumor initiation would be larger than that currently accepted. A further consequence of the present hypothesis is that preventing virus reactivations, where possible by pharmacologic prophylaxis or medical modulation of the immune response, should counteract cancer development.

TESTING THE HYPOTHESIS

The demonstration that genetic and epigenetic damage occurs in latently infected cells and that some damaged cells survive in the setting of natural infections is crucial in validating our hypothesis. It would therefore be important to investigate the process whilst it is occurring. It is conceivable to plan prospective studies of patient populations at risk for recurrent or persistent viral infections. The genetic integrity of cells latently or persistently infected by a given virus could be studied using methods applicable to a large number of samples and correlated with virus shed at the site of sampling. For example the analysis of DNA damage could be associated with HPV isolation at the time of pap test screening, or with EBV viral load determination in the follow-up of transplant patients[28]. Retrospective and prospective studies could be implemented, analyzing possible correlation between frequency of different virus reactivations, severity of these reactivations, evidence of genetic damage in cells that harbor latent viruses and development of malignancies, in order to better define the importance of evocative findings[56]: ideal candidates for these studies would be populations of immunocompromised patients such as those in post-transplant settings[57]. Chronic infections, clinically manifest or subclinical, are an additional interesting condition for virus related DNA damage investigation[37]. In this setting the measurement of chromosomal abnormalities in peripheral blood lymphocytes should result particularly fruitful, if one considers that circulating cells are exposed to infectious agents even in localized infections during tissue perfusion[23,58,59].

Precious information would be generated through the analysis of pre-tumoral and tumoral banked samples, where the observation of abnormal mitosis and genetic abnormalities can be associated with the identification of virus related antigens or nucleic acids[60], while prospective studies could include virus isolation. As an example, hepatic biopsies from non-responders to anti-HCV treatment could be analyzed for the presence of genetic abnormalities and the findings would be compared to responders in relation to incidence of hepatocarcinoma development over time. *In vitro* studies should be devised choosing experimental settings that guarantee the closest simulation of authentic *in vivo* situations, cautiously choos-

ing animal models and transformed cell lines, avoiding non human cell cultures, and laboratory strains of viruses[61]. Ideally fresh clinical virus isolates should be used to infect cells that are the authentic sites of latency *in vivo*, looking for consequences of virus infection on mitosis, chromosome integrity and the epigenetic stage.

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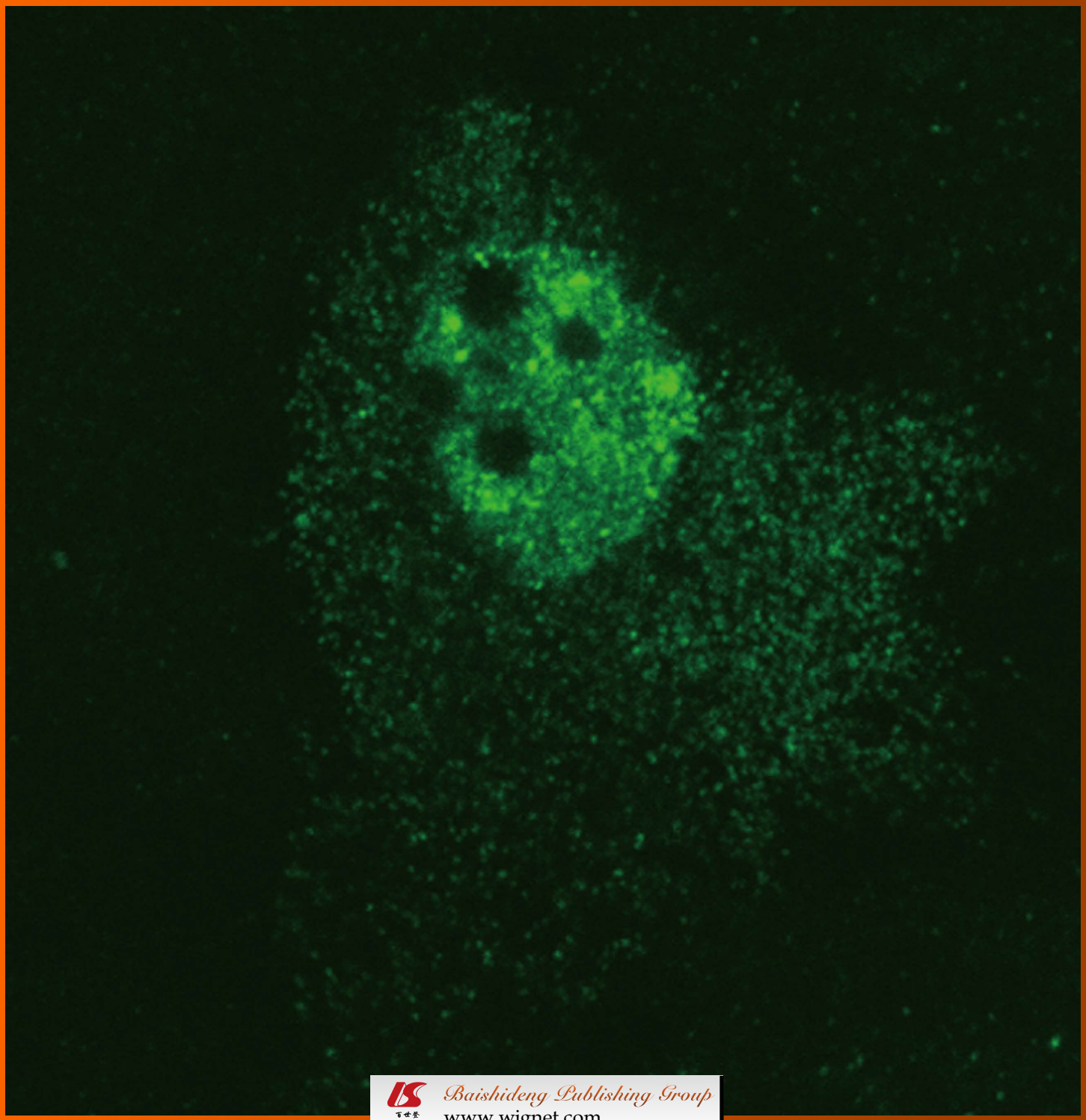

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REVIEW

Nuclear domain 10 of the viral aspect

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Abstract

Nuclear domain 10 (ND10) are spherical bodies distributed throughout the nucleoplasm and measuring around 0.2-1.0 μm. First observed under an electron microscope, they were originally described as dense bodies found in the nucleus. They are known by a number of other names, including Promyelocytic Leukemia bodies (PML bodies), Kremer bodies, and PML oncogenic domains. ND10 are frequently associated with Cajal bodies and cleavage bodies. It has been suggested that they play a role in regulating gene transcription. ND10 were originally characterized using human autoantisera, which recognizes Speckled Protein of 100 kDa, from patients with primary biliary cirrhosis. At the immunohistochemical level, ND10 appear as nuclear punctate structures, with 10 indicating the approximate number of dots per nucleus observed. ND10 do not co-localize with kinetochores, centromeres, sites of mRNA processing, or chromosomes. Resistance of ND10 antigens to nuclease digestion and salt extraction suggest that ND10 are associated with the nuclear matrix. They are often identified by immunofluorescent assay using specific antibodies against PML, Death domain-associated protein, nuclear dot protein (NDP55), and so on. The role of ND10 has long been the subject of investigation, with the specific connection of ND10 and viral infection having been a particular focus for almost 20 years. This review summarizes the relationship of ND10 and viral infection. Some future study directions are also discussed.

Key words: Nuclear domain 10; Promyelocytic Leukemia; Speckled protein of 100 kDa; Death domain-associated protein; Virus; Viral replication

Core tip: We, for the first time, discussed the function of nuclear domain 10 (ND10) as a nuclear structure. Although the ND10 components, especially Promyelocytic Leukemia bodies, Speckled Protein of 100 kDa and death domain-associated protein, have been widely investigated for their roles in viral gene expression and viral replication, individual virus interacts with ND10 differentially as we summarized up in this review. This review is expected to guide readers especially virologists and cell biologists to understanding the interaction of ND10 with viruses.

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ND10: GENERAL INFORMATION

Mammalian cells contain differentially functional compartments called organelles, which are separated from the cytoplasm by a lipid bilayer membrane. The nucleus is an extremely dynamic organelle and highly organized compartment with multiple functions (reviewed in Dundr

et al[1], Dundr *et al*[2], and Zhao *et al*[3]) the nucleoplasm consists of soluble and insoluble materials that keep the genomic structure intact and host the complicated process of gene transcription. Some insoluble and soluble materials congregate together to form shaped structures such as nuclear domain 10 (ND10 can also refer to nuclear dot 10)[4]. When analyzed by indirect immunofluorescence microscopy, many nuclear proteins are seen to localize in distinct structures with punctate staining patterns[5,6]. Nuclear structures, such as speckles, paraspeckles, nucleoli, Cajal bodies, polycomb bodies, and ND10, are formed primarily by protein-protein, protein-RNA, or protein-DNA interactions[1]. Each nuclear body has a matrix protein that is essential for the formation of the specific nuclear body. ND10 are subnuclear structures that gather many different SUMOylated nuclear proteins (such as Daxx and SP100). The formation of ND10 depends on Promyelocytic Leukemia (PML) protein. Past observations confirm that PML knockout cells lack ND10 and that transfecting exogenous PML into PML knockout cells results in the restoration of ND10[7,8]. Most DNA viruses replicate DNA and transcribe genes in the nucleus after their genomic DNA enters the nucleus by facilitated transport through the nuclear pore complex[9]. Once inside the nucleus, viral genomes distribute randomly, but it appears that only those at ND10 replicate and transcribe predominantly[10-13], suggesting specifically that the environment at ND10 is particularly advantageous for the virus. However, the ND10 proteins [such as PML, Speckled Protein of 100 kDa (SP100), and Daxx] are interferon-upregulated and have repressive effects on viral replication[14-25]. Moreover, most DNA viruses encode an immediate-early protein that induces the dispersion of ND10[10,26-29], and in the absence of these viral proteins, replication is severely retarded[13,29,30]. These findings have led to the hypothesis that ND10 are also part of nuclear defense mechanism[4]. At this point, the effects of ND10 on viral replication remain to be settled.

A HISTORIC OVERVIEW OF ND10

There are five hallmark events in the history of studying ND10. First, a French paper in 1960 described an unknown nuclear structure in rabbit cells with the Papillomavirus as an electron-dense body[31]. It was the earliest description of the nuclear structure, but it left everything unexplained, other than providing that observation. Second, there was not any other information that could lead to a deeper investigation of these nuclear structures until they were first identified (by immunofluorescence analysis, using specific antibodies that were later revealed to be against SP100 and NDP55) as ND10 in 1991 by Ascoli *et al*[32]. SP100 was later proved to be essential for the formation of ND10[33]; NDP55 has not been characterized so far. Ascoli *et al*[32] investigated the structure in different types of cells. A combination of immunofluorescence analysis and electronic microscopy confirmed that ND10 are the structures that were previously observed in 1960. Third, in the process of investigating the function of ND10, ND10 were found to be related to herpes simplex virus type 1 (HSV-1) infection[26,34] in 1993 and 1994; these studies (by Maul GG, the Wistar Institute, the United States of America, and Everett RD, the MRC Virology Unit, Glasgow, United Kingdom) awakened the interest of virologists with regard to the interaction of ND10 and many different viruses. The interactions of ND10 and a variety of viruses will be discussed in this review. Fourth, it was determined that PML knockout mice lack ND10, which provided direct evidence that supported the hypothesis that the protein PML is essential to the formation of ND10. It was confirmed, as well, by the experimental results that demonstrate that the transfection of PML into PML-/- cells restores ND10[26,35]. PML-/- mice live normally, which further obscured the function of PML, though later studies were able to determine that PML-/- have a greater tendency to develop cancer than do their PML+/+ counterparts[36]. Fifth, ND10 components were identified. Even though more than 60 nuclear proteins have been shown to be more-or-less related to ND10[37,38], three components are thought to be the primary ND10 proteins (called the prototype proteins of ND10): PML, Daxx, and SP100. Other important events relating to the study of ND10 will be discussed in the following sections.

MOLECULAR ASPECT OF ND10

Promyelocytic leukemia

The molecular mechanism of the biogenesis of ND10 was a complete mystery until PML was identified as forming the matrix of ND10. PML is a tumor-suppressor protein that in both humans and mice is encoded by the PML gene. This gene was found to be involved in translocation with the retinoic acid receptor alpha (*RARalpha*) gene, causing acute promyelocytic leukemia (APL) (see the review by de Thé *et al*[39]). The protein encoded by this gene was therefore named after PML. PML is also called tripartite motif (TRIM) 19 because it is a member of the TRIM family[40]. The TRIM motif includes three zinc-binding domains, a RING, two B-boxes, and a coiled-coil region. Phosphorylation is required for the high SUMOylation of PML; SUMOylated PML localizes to ND10, where it functions as a transcription factor and tumor suppressor[41]. Its expression is cell-cycle related; therefore ND10 morphology and number in the nucleus are dependent on the cell cycle[42]. It regulates the p53 response to oncogenic signals, which might explain how the translocation of PML with RARalpha causes APL. Right after its identification, ND10 were shown to be important in cell differentiation and cell growth; this was first indicated in studies of promyelocytes from patients suffering from APL[43-45]. In the promyelocytes from these patients, ND10 cannot be detected. When cells are treated with retinoic acid (RA) or with arsenic trioxide, ND10 are restored and the APL phenotype is reversed and the patients can be cured with these agents (reviewed by Melnick and Licht[46]). PML has about 11 isoforms that are caused by the extensive

alternative splicing of this gene. PML isoforms vary in the protein's central and C-terminal regions; all variants encode the same N-terminus[47]. Some isoforms of PML are cytoplasmic, but most of the isoforms are nuclear proteins important for ND10 formation.

Speckled protein of 100 kDa

SP100 was first identified by immunofluorescence using autoantibodies from patients with primary biliary cirrhosis, and its cDNA was then isolated and cloned; it was found to encode a human nuclear antigen distributing in the nucleus as speckles[48]. SP100 is a single-copy gene sited in human chromosome 2q37 and, like PML, it is IFN upregulated. The *SP100* gene needs to be spliced and gives rise to a number of speckled protein of 100 kDa (SP100) isoforms: SP100A, -B, -C, and -HMG[48-52]. The four SP100 isoforms share a homologous 476 N-terminal amino acid, but differ in their C-terminal part. The most abundant isoform is SP100A, which has 480 amino acids and migrates to 100 kDa on SDS-PAGE[51]. SP100A most likely does not bind to DNA alone because it lacks all other domains of SP100B, -C, and -HMG. It may be recruited to DNA *via* association with DNA-binding proteins such as hHMG2/DSP1[53], the B-cell-specific transactivator Bright[54], or ETS-1[55]. SP100B contains a SAND domain (SAND stands for SP100, AIRE, NucP41/75, and DEAF1), SP100HMG contains a SAND domain and an HMG box, and newly described SP100C contains SAND, PHD, and Bromo domains[52,56]. SP100 is one of the prototypical proteins of ND10, and it colocalizes with Daxx and PML in ND10. SP100B, -C, and -HMG isoforms contain SAND, PHD, Bromo, and HMG domains and are highly SUMOylated. All the domains are suggestive of a role in chromatin-mediated gene regulation. The three minor isoforms contain a SAND domain that binds to DNA and is required if SP100 is to have transcriptional regulating activity.

Death domain-associated protein

Upon its discovery, death domain-associated protein (Daxx beta) was found to be a protein of the classical death receptor[57]. It was found to bind specifically to the Fas death domain *via* its C-terminal portion. Overexpression of Daxx enhances Fas-mediated apoptosis through activating the Jun N-terminal kinase (JNK) pathway. It was later found that Daxx interacted with CENP-C, one of the few known intrinsic proteins of the human centromere[58]. CENP-C is thought to play structural as well as regulatory roles crucial to proper chromosome segregation and mitotic progression. The interaction between CENP-C and Daxx was then confirmed by an immunofluorescence assay that found the colocalization of these two proteins at discrete spots in the nuclei of some interphase cells[58]. The other Daxx-binding proteins include the transcription factor Pax3[59] and DNA methyltransferase I [60]. They both are related to centromeres such as CENP-C and are not related to ND10. Therefore, Daxx is a protein of centromere. However, Ishov *et al*[7] found that PML recruited Daxx to ND10. Interestingly, in PML-/- cells, Daxx totally stays in the centromere. Therefore, Daxx might travel from centromere to ND10 or from ND10 to centromere. Ishov *et al*[42] also found that Daxx and the SWI/SNF protein ATRX are both associated with two intranuclear domains: ND10 and heterochromatin. The accumulation of ATRX at ND10 was mediated by its interaction with the N-terminus of Daxx. Although ATRX was present in heterochromatin during the entire cell cycle, Daxx was actively recruited to this domain at the end of the S-phase. Daxx functions as an adapter for ATRX accumulation at ND10[42]. Daxx can be highly SUMOylated, and SUMOylation was found to be crucial for targeting Daxx to PODs and for the transrepression of several SUMOylated transcription factors, including the glucocorticoid receptors (GR)[61]. Recently, two variants of Daxx were identified. The two novel variants of Daxx were termed Daxx- and Daxx-γ, and these variants are generated by alternative splicing. They have a truncated regulatory C terminus, and Daxx- and Daxx-γ show markedly decreased affinities to PML and have a different nuclear distribution[62].

In summary, all three of the prototypical proteins (PML, SP100, and Daxx) of ND10 share some similar characteristics: (1) They colocalize with ND10. Their colocalization in ND10 depends on PML[7,8], and SP100 can also affect ND10 formation[33]. These proteins are prototypical components of ND10; (2) They can be up regulated by interferon, which provided the first evidence to support the hypothesis that ND10 are defensive against viral infection[4]; (3) The prototypical proteins of ND10 are all highly SUMOylated, SUMOylation is important for the formation of ND10, Daxx function, and the interaction of the three proteins; (4) They are all cancer gene repressors. Although PML-/- mice can still live normally, they are shown to have a higher chance of developing cancer[63]; (5) All three genes produce different isoforms *via* alternative splicing; and (6) They are all viral replication inhibitors, which will be discussed in the review below.

ND10 function

ND10 came to the forefront because it was found that t(15; 17) translocation causes the fusion of PML and RARA (generating PML/RARA) and the dysfunction of both PML and RARA (consequently resulting in APL). The oncogenic PML/RARA protein disrupts ND10 in a reversible manner upon being treated with retinoic acid and/or arsenic, either of which treatment can cure the patients with APL[64-68]. ND10 number and size are regulated in several cellular responses: viral infection[69], DNA-damage, transformation[70-72], and oxidative stress[73,74]. The transcriptions of PML, SP100, and Daxx are dramatically enhanced by interferons. However, PML-/- mice develop normally and live well without the formation of ND10, demonstrating that ND10 are not required for most basic biological functions. Nevertheless, recent data have implicated PML in the control of cellular senescence and stem

cell self-renewal, extending the fields of the investigation of PML function[75,76].

ND10 studies have been so intense in recent years that novel information about these structures is being uncovered continuously; however, the function of PML bodies is still not fully understood. Three models have been proposed: the Depot or Sequestration model; the Hotspot model, and a site of specific nuclear activities. These models are described in the following paragraphs.

Depot or sequestration model

The nuclear domains are proposed to be aggregations of excess nucleoplasmic protein[77]. This model suggests that the ND10 components in the nucleoplasm have a dynamic nature, that is, they move from ND10 to the functional sites where they are needed. In other words, the aggregated proteins in ND10 are sequestered. This sequestration is evidenced by the fact that the PML partners in ND10 vary significantly between individual partners and levels of PML expression, as well as SUMOylation. A well-studied sequestered component in ND10 is Daxx, a potent repressor that forms partitions between SUMOylated proteins, including PML and many transcription factors. Sequestration of Daxx by ND10-associated, SUMOylated PML releases transcriptional repression by DNA-bound SUMOylated transcription factors[61,78-80].

Hotspot model

This model proposes that ND10 are the sites of the post-translational modification and the degradation of proteins. It is supported by the facts that SUMO-1 molecules aggregate in ND10 and ND10 might be the hot sites for SUMOylation, that the acetylation and phosphorylation of p53 at PML bodies enhance the activity of p53[16,81,82], and that the 19S and 20S proteasome subunits localize at some PML bodies[83].

Third model

Proposes ND10 to be sites of specific nuclear activities, such as transcriptional regulation and DNA replication. This model is supported by the detection of nascent RNA around ND10[84], the association of ND10 with regions of high transcriptional activity[85], and the non-random nature of PML body assembly (based on the conservation of their size and position) following dissociation and re-formation as a result of cellular stress[86].

INTERACTIONS OF ND10 AND VIRUSES

Herpesviruses

Human herpesviruses are divided into three subfamilies: alpha, beta, and gamma. The alpha subfamily includes Herpes Simplex Virus 1 and 2 (HSV-1 and HSV-2) and the Varicella Zoster Virus (VZV). The beta subfamily has cytomegalovirus (CMV) and human herpesvirus 6 and 7 (HHV-6 and HHV-7). Kaposi's sarcoma-associated herpes virus (KSHV) and the Epstein-Bar virus (EBV) are in the gamma subfamily. These viruses are characterized by their practice of setting up latency in the host after primary infection. After entering to the nucleus through nuclear pores, these DNA viruses replicate their DNA and transcribe their genes inside the nucleus, preferably at ND10[4]. Therefore, the interaction of ND10 and herpesviruses occurs at the very early stage of infection.

Herpes simplex

The first virus found to be connected to ND10 was herpes simplex (HSV)-1. In 1993, Maul *et al*[28] were the first to discover that Vmw110 (ICP0-infected cell protein 0) localizes to ND10. Interestingly, they also showed that the C-terminal portion of ICP0, when linked to a heterologous protein, disrupts the normal distribution of PML. These observations presented the first link between processes involved in the control of cell growth and viral infection and latency. Later, Maul and Everett[11,26,34,87] systemically collaborated on the investigation of the interaction of ND10 and HSV-1, which collaboration typically combined the views from a cell biologist (Maul) and a virologist (Everett) on the direction to revelation of the phenomenal interaction of viral molecules and ND10. This, according to the authors' opinion, could be the most important contribution to the ND10 field.

It is now known that ICP0 disrupts ND10 through mediating the loss of the SUMO-1-modified forms of PML and the subsequent proteasome-mediated degradation of the PML protein[14-15,88-90]. The results were consistent with the finding that PML residue lysine 160 is the SUMOylation site and the mutation of this residue makes PML resistant to degradation by ICP0[91]. ND10 function might not be so critical for HSV-1 lytic infection because ICP0-deleted HSV-1 can replicate well, especially at a high multiplicity of infection (MOI).

It was visualized that both parental and replicated HSV-1 amplicon genomes were in association with ND10 in live cells[92]. It is likely that the genomes situated at ND10 preferentially form viral replication compartments. Tang *et al*[12] further figured out that there exist minimal viral DNA sequences and viral proteins that are essential and sufficient for the replication of DNA and the transcription of RNA at ND10 by the virus. For HSV-1 we found that a specific viral DNA sequence, OriS, and the viral immediate-early proteins ICP4 and ICP27 are sufficient for a reporter gene placed in cis at the OriS sequence to transcribe RNA at ND10[12]. HSV-1 DNA replication results in formation of compartments in the nucleus; it has been shown that some, but not all, PML isoforms are recruited to the replication compartments[93]. Viral DNA replication compartments also contain many other viral and cellular proteins that have different functions, many of which are required for DNA replication, DNA repair, and DNA stabilization[94]. However, the function of ND10 proteins in the DNA replication compartments is not fully understood.

HSV-1 with deleted ICP0 has an obvious defect in viral gene expression and plaque formation in limited-pas-

sage human fibroblasts (though not in mouse fibroblast cells)[95,96]. This suggests both that ND10 have defensive effect on HSV-1 infection and that ICP0 can abolish the defensive effect of ND10 in human fibroblasts. ICP0 is a RING finger E3 ubiquitin ligase that induces the degradation of PML. Depletion of PML from human fibroblasts increases ICP0-null mutant *HSV-1* gene expression but not to wild-type levels[96]. Another major ND10 protein, SP100, has a similar effect on ICP0-deleted HSV-1 gene expression[96]. It has been shown that all four SP100 isoforms stabilize ND10 and protect PML from ICP0-based hydrolysis[18]. Depletion of either all PML isoforms or all SP100 isoforms reduces the other constituent ND10 protein, suggesting that different ND10 proteins use different mechanisms to inhibit virus infection at the immediate-early stage of HSV-1 infection[18]. Simultaneous depletion of both PML and SP100 proteins complements the mutant virus to a greater degree, implying that PML and SP100 could have additive or synergistic effects on viral replication[96].

HSV-1 ICP0 might be important for the activation of lytic infection and the countering of the cell-mediated repression of viral gene expression by HSV-1. This repression is defended by preexisting cellular proteins, and those proteins function as intrinsic antiviral resistance or intrinsic defense. PML and SP100, as we discussed above, are two of the core components of ND10 and contribute to intrinsic resistance. But how about other ND10 proteins, such as, ATRX and Daxx? ATRX and Daxx are known to comprise components of a repressive chromatin-remodeling complex. It has been shown that the infection of ICP0-deleted HSV-1 (not wild-type HSV-1) can replicate at a greater level in both ATRX- and hDaxx-depleted cells than it can in normal cells[97], suggesting that ATRX and hDaxx act as a complex to play intrinsic antiviral resistance to HSV-1 infection, which is counteracted by ICP0.

Cytomegalovirus

Cytomegalovirus (CMV) infection differs from that of HSV-1 in host range and replication. HCMV can infect only human cells productively and causes diseases in humans only, and it replicates slowly in cell culture. HCMV is similar to HSV-1 in many ways: (1) setting up latency after primary infection in host; (2) sequential viral gene expression; and (3) viral DNA replication at ND10, preferentially. Following the studies of ND10 and HSV-1 interaction, many ND10 components have been demonstrated to have a repressive effect on CMV gene expression and viral replication (reviewed by Saffert and Kalejta[98]). The first ND10 protein investigated for its role in *HCMV* gene expression and viral replication was Daxx. In that study, Daxx was found to interact functionally with HCMV tegument protein pp71[16]. The Stamminger group[99] also investigated PML to see whether PML could have any effects on viral gene expression or on viral replication. After comparing HCMV replication in PML-kd or hDaxx-kd cells with that in normal cells, they revealed that immediate-early (IE) gene expression increased to a similar extent, regardless of whether PML or Daxx was depleted[98]. Their experimental results suggest that PML and Daxx might function using different mechanisms to suppress HCMV replication; double-knockdown cells depleted of both PML and hDaxx support the additive enhancement of HCMV infection in the replication efficacy of HCMV compared to that of single-knockdown cells[99]. Finally, they also found that the infection of SP100 knockdown (kd) cells with HCMV resulted in a significantly increased plaque-forming ability[99,100].

Like HSV-1, HCMV infection can also disrupt ND10, but the mechanisms of dispersing ND10 might be different. HSV-1 ICP0 induces the loss of the SUMO-1-modified forms of PML and the proteasome-mediated degradation of the PML protein[14,15,88-90]. However, in CMV-infected cells, PML is not degraded[13,101]. For cytomegaloviruses (including MCMV and HCMV), IE1 has been identified to disperse ND10 by an as yet unknown mechanism, but it is not able to degrade PML[27,101-104]. HCMV IE1's induction of PML deSUMOylation, reported by Lee *et al*[101], needs to be investigated for MCMV IE1.

Species-specificity is one of the major characteristics of cytomegaloviruses (CMVs) and is the primary reason for the lack of a mouse model for the direct infection of human CMV (HCMV). It has been determined that CMV cross-species infections are blocked at the post-entry level by intrinsic cellular defense mechanisms[105,106], but few details are known. We discovered that ND10 of human cells is not disrupted by murine CMV (MCMV) and that the ND10 of mouse cells is not disrupted by HCMV[107], although the ND10-disrupting protein, immediate-early protein 1 (IE1), also colocalize with ND10 in cross-species infections[107]. In addition, we found that the UL131-repaired HCMV strain AD169 (vDW215-BADrUL131) can infect mouse cells to produce immediate-early (IE) and early (E) proteins but that neither DNA replication nor viral particles are detectable in mouse cells. Unrepaired AD169 can express only IE1 in mouse cells. In both HCMV-infected mouse cells and MCMV-infected human cells, the knocking-down of ND10 components (PML, Daxx, and SP100) resulted in significantly increased viral-protein production. Our observations provide evidence to support our hypothesis that ND10 and ND10 components might be important defensive factors against CMV cross-species infection.

EPSTEIN-BARR VIRUS AND KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

The relationship of Epstein-Barr Virus (EBV) or Kaposi' s sarcoma-associated herpesvirus (KSHV) with ND10 has been less investigated than has that of HSV-1 or CMV (with ND10). The first study of the interaction of EBV and ND10 also came from the Maul group. Bell *et al*[108] studied the effect of the EBV on ND10, and its (EBV's) spatial distribution in the nucleus of cells during latency and lytic reactivation. In EBV, latently-infected Burkitt's lymphoma,

lymphoblastoid, and D98/HR1 cells, ND10 were intact. Fluorescent *in situ* hybridization (FISH) revealed no association between viral episomes and ND10 during latency, implying that the maintenance replication of EBV, which depends on host cell proliferation, occurs independently of ND10. Upon lytic activation, ND10 become dispersed in cells expressing lytic proteins. Thus, latency does not require or induce the interaction of EBV and ND10 for transcription or replication, whereas lytic replication triggers the dispersion of ND10 proteins and occurs in close association with PML aggregates. The required movement of chromosome-attached latent EBV episomes to ND10 after reactivation from latency might include the physical release of chromosome-bound episomes. Only episomes that come in contact with ND10 after such a release might be able to begin the process of lytic replication[108]. The dispersion of ND10 by EBV in lytic infection might be through molecular and functional interactions between the EBV BZLF1 protein and the PML[109].

There are many fewer functional studies of ND10 proteins in EBV infection or reactivation than there are of those proteins in HSV-1 or CMV. So far, SP100 appears to be an effective ND10 protein that is related to *EBV* gene expression and viral reactivation. The EBV EBNA-LP protein is a potent gene-specific coactivator of the viral transcriptional activator, EBNA2. Ling *et al*[17] found that EBNA-LP interacts with ND10 protein SP100 and displaces SP100 and heterochromatin protein 1alpha (HP1alpha) from ND10. Their experimental results suggest that SP100 is a major mediator of EBNA-LP coactivation[17]. Recently, Tsai *et al*[110] showed that the EBV major tegument protein BNRF1 interacts with host-cell ND10 proteins and promotes viral early gene activation. Specifically, they demonstrated that BNRF1 interacts with the Daxx at ND10 and interferes with the formation of the Daxx-ATRX chromatin remodeling complex. Furthermore, the knockdown of Daxx and ATRX induces the reactivation of EBV from latency in infected lymphoblastoid cell lines, suggesting that Daxx and ATRX play a role in the regulation of viral gene expression and viral replication.

KSHV interacts with ND10 at the very early stage after reactivation. Although EBV and KSHV are so similar in many aspects that they are classified into the gama-herpesviral subfamily, they are different in many other characteristics. For example, KSHV might not be able to disrupt ND10, even though that particular claim is arguable. Wu *et al*[111] first studied the interaction of ND10 and KSHV and found that the KSHV protein, K8, interacted with PML; nevertheless, they clearly demonstrated that KSHV infection (latent or lytic) cannot disrupt ND10[111,112]. Our unpublished data also support their conclusion that ND10 are not dispersed by KSHV infection. However, this has been recently challenged by other studies[111,112]. In one such study, Marcos-Villar *et al*[113] stated that the KSHV protein LANA2 increased the levels of SUMOylated PML and induced the disruption of ND10 by a proteasome-mediated mechanism. They also reported that ND10 disruption needs both the integrity of a SUMO interaction motif (SIM) in LANA2 and the lysine 160 from PML. Moreover, they showed that the depletion of LANA2 in PEL cells led to an increase in the PML levels[111,112]. Arguably, KSHV's dispersion of ND10 was not clearly shown in the published pictures. Interestingly, the authors didn't cite the paper by Wu *et al*[111] that is intimately related to the subject.

As for the molecular and functional interaction of KSHV and ND10 proteins, only a few publications have been presented. First, Murakami *et al*[114] reported that Daxx is a LANA-binding protein and that interaction made LANA inhibit the repressive effect of Daxx on VEGF expression. Their results suggest that LANA contributes to the high expression of the vascular endothelial growth factor (VEGF) receptors in KS lesions by interfering with the interaction of Daxx and Ets-1[114]. Other studies showed the existence of an interaction between PML and KSHV proteins (including K8 and LANA2)[111,115]. The biological significance of this interaction is still unclear.

OTHER DNA VIRUSES

Adenovirus

Adenovirus (Adv) is another virus that interacts with ND10. It was found that Adv infection changed the morphology of ND10 from being spherical punctate structures to being fibrous ones. This morphological change is caused by the molecular interaction of the Adv protein, E4 ORF3, and PML[116]. The other Adv protein found to interact with PML was E1A, which is an oncoprotein[116]. This study suggests that PML in ND10 might be involved in the cancerous consequence of Adv infection. More recently, a study by Hoppe *et al*[117] showed the PML isoform interacting directly and specifically with Adv E4 Orf3 *in vitro* and *in vivo*. Moreover, Hoppe *et al*[117] reconstructed ND10 in PML-null cells by inducing the transient transfection of different PML isoforms. They observed that only those ND10 formed from PML isoform Ⅱ were morphologically changed by E4 Orf3. Their data suggest that the interaction of E4 Orf3 and PML isoform Ⅱ is required for ND10 rearrangement[117].

The E4 ORF3 protein is required for Adv DNA replication when the cells are in the interferon (IFN)-induced antiviral state. ND10 prone proteins are all IFN-upregulated. This may reflect the fact that PML, Daxx, and SP100 are encoded by an interferon-stimulated gene. If so, can the interaction of E4 ORF3 and ND10 have any effect on Adv replication or viral gene expression? Ullman *et al*[118] demonstrate that the interaction of E4 ORF3 and ND10 antagonizes an innate antiviral response mediated by both PML and Daxx. Depleting any one of these proteins makes it possible to restore the replicative capacity of the virus using the E4 ORF3 protein deleted in the IFN-induced antiviral state. The interaction of Adv and ND10 has been also investigated with respect to SP100. Obviously, SP100 SUMOylation

is also affected by E4 ORF3, which in part contributes to the morphological change of ND10[89]. We think that it is critical to investigate whether E4 ORF3's interaction with ND10 plays a role in the oncogenesis of Adv. The interaction of E1A (an oncoprotein of Adv) and ND10 might be more important in the field of ND10 and viruses.

Human papillomavirus

Using indirect immunofluorescence in combination with fluorescence in situ hybridization, Swindle *et al*[119] found that human papillomavirus (HPV) DNA replication is targeted to host nuclear domains that are active during the late S phase, when such domains are limited in number. It was also observed that E1 and E2 partially or completely colocalize with ND10. The observation suggests that HPV DNA amplification might be partially coupled to virion assembly[119]. Interestingly, Florin *et al*[120] showed that the minor capsid protein L2 of HPV interacted with ND10-associated proteins. They observed that (1) the PML was unaffected by L2; (2) SP100 was released from ND10 upon L2 expression; and (3) In contrast to SP100, Daxx was recruited to ND10 by L2 expression. These studies suggested that ND10 might be involved in HPV capsidation.

Simian virus 40

Simian virus 40 (SV40) is a small DNA virus. Like other DNA viruses, SV40 starts transcription and replication adjacent to ND10. In an early study, we identified a specific viral DNA sequence and its binding protein that determine the location of these synthetic activities at such restricted nuclear sites[121]. A beta-galactosidase gene was introduced into an expression vector that contains partial and overlapping SV40 sequences. Transcripts derived from control plasmids were found throughout the nucleus and at highly concentrated sites but not at ND10. SV40 genomic segments supported ND10-associated transcription only when the origin and the coding sequence for the large T antigen were present. When the large T-antigen coding sequence was eliminated but the T antigen was constitutively expressed in COS-7 cells, the viral origin was sufficient to localize transcription and replication to ND10. Large T antigen expressed from plasmids without the viral core origin did not bind or localize to ND10. Blocking of DNA replication prevented the accumulation of transcripts at ND10, indicating that only sites with replicating templates accumulated transcripts. Transcription at ND10 did not enhance total protein synthesis of plasmid transcripts. These findings suggest that viral transcription at ND10 may only be a consequence of viral genomes directed to ND10 for replication. Although plasmid transcription can take place anywhere in the nucleus, T-antigen-directed replication is apparently restricted to ND10[121].

RNA VIRUSES

The first RNA virus studied for its interaction with ND10 was the lymphocytic choriomeningitis virus (LCMV), a single strand RNA virus, but interestingly, the interaction occurs in the cytoplasm. In cells infected with LCMV, the viral zinc-finger (Z) protein forms large bodies primarily in the cytoplasm. Z protein can redistribute PML from the nucleus to the cytoplasm, and PML and Z protein colocalize in the cytoplasm[35,122]. The similar function of Z protein was also found in other viruses of Arenaviridae[35,122]. The interaction of PML and Z proteins may influence certain unique characteristics of arenavirus infection.

Another RNA virus is hepatitis delta virus (HDV). HDV is a single-stranded RNA virus and has only one coding region producing the hepatitis delta antigen (HDAg). HDAg is expressed in two isoforms, small (S-HDAg) and large (L-HDAg). S-HDAg is required for the replication of HDV, while L-HDAg inhibits viral replication and is required for the envelopment of the HDV genomic RNA by hepatitis B virus proteins[123]. Bell *et al*[124] found that over half of the L-HDAg domains were localized beside ND10. At later times, ND10-associated proteins such as PML were found in larger HDAg complexes, in which PML was found chiefly in the rims of the spheres. Other ND10 components (SP100, Daxx, and NDP55) were found in the centers of the spheres. HDV genomic RNA was distributed more uniformly throughout the nucleus, but nascent viral RNA colocalizes with L-HDAg and the transcriptional repressor PML. These results suggest that this RNA virus, like DNA viruses, can alter the distribution of ND10-associated proteins and preferably transcribe mRNA at ND10. It is not clear whether the ND10-associated proteins (PML) play a role in the regulation of HDV RNA synthesis.

As for human immunodeficiency virus (HIV), the results have been controversial. Bell *et al*[125] reported that no significant relationship was observed between ND10 or any of the following: HIV-1 DNA, transcription foci, and integrated DNA. Their results showed that HIV-1 did not modify ND10 at early or late times of infection[125]. However, Turelli *et al*[126] reported that incoming retroviral preintegration complexes trigger the exporting-mediated cytoplasmic export of PML. They further described how the HIV genome associates with PML before nuclear migration. Further experiments are needed to reveal the detailed interaction of HIV and ND10.

VIRAL DISPERSION OF ND10

During viral infection, viruses and ND10 interact differently. The modification of ND10 structure can include (1) an increase in the size and number of ND10 per nucleus by double strand RNA viruses because their infection can induce IFN; (2) a change to the shape of ND10; (3) a decrease in the size or the number (of ND10) per nucleus; and (4) a total dispersal of ND10. Only CMV, EBV, and HSV have been clearly shown to disperse ND10. Here we take the MCMV infection as an example to show the real dispersing of ND10 (Figure 1). As we stated above,

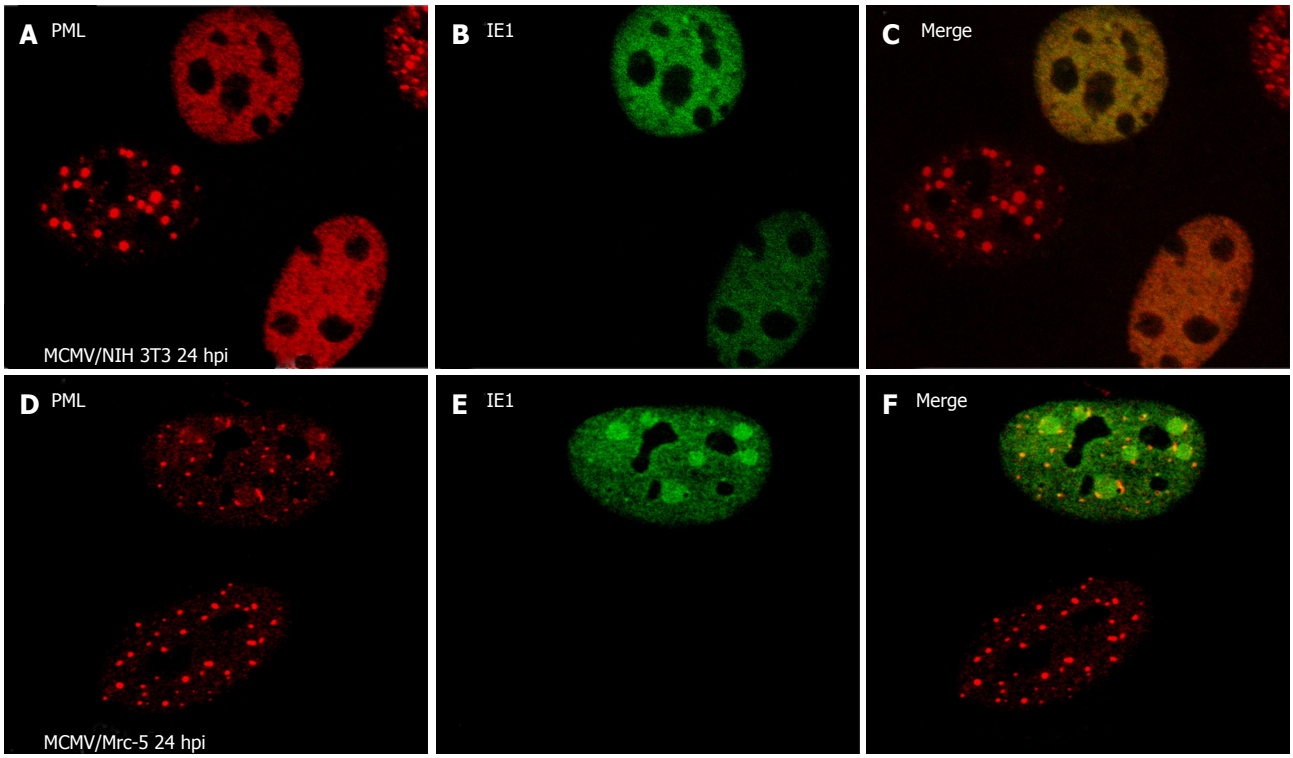

Figure 1 Immunofluorescent assay to show cytomegalovirus infection and nuclear domain 10. A: After murine cytomegalovirus (MCMV) infection in NIH3T3 cells for 24 h, cells were stained with anti-Promyelocytic Leukemia bodies (PML) antibody (rabbit) to show nuclear domain 10 (ND10) (in red); B: Anti-IE1 antibody (mouse) was used to show IE1 (in green); C: The merged picture is shown in; D: After MCMV infection in Mrc-5 cells for 24 h, cells were stained with anti-PML antibody (rabbit) to show ND10 (in red); E: Anti-IE1 antibody (mouse) was used to show IE1 (in green); F: The merged picture is shown.

CMV infection is species-specific. Interestingly, we discovered that murine CMV (MCMV) infection in human cells cannot disperse ND10[107], suggesting the involvement of ND10 in species-specificity. We performed comparative IFA to analyze the ability of IE1 to disperse ND10 in cross-species-infected cells as opposed to in natively infected (mouse) cells. We infected wt-MCMV into both Mrc-5 cells and NIH3T3 cells for 24 h. Cells were fixed and permeabilized and stained with anti-PML to show ND10 (red, Figure 1A, D) and with anti-IE1 to show the distribution of IE1 (green, Figure 1B, E). As can be seen in the MCMV-infected mouse cells, IE1 was diffusely distributed in the nucleus at 24 hpi. Interestingly, the IE1 of MCMV formed domains (Figure 1E) in human cells and lost the ability to disperse ND10, their distribution being different from that found in MCMV-infected mouse cells (Figure 1A-C). There is no standard for judging the level of viral effect on ND10 structure because ND10 number or size can vary in different cell cycles. Therefore, one has to be careful to make conclusions of dispersing or disrupting ND10 by any viral infection or transient transfection.

FUTURE DIRECTIONS FOR INVESTIGATIONS INTO THE VIRAL ASPECT OF ND10

A great deal of progress regarding the interaction of ND10 and viruses has been made in the past decades. A lot of questions are still left behind us, which makes the future direction of studies in the ND10-viruses field: (1) ND10 structure and ND10 protein are clearly related to cancer development (at least to some types of cancers). Therefore, the interaction of tumor viruses and ND10 should be the future focus of research in this field; (2) ND10 aggregate a lot of nuclear proteins that have different functions; we already know that SUMOylation is important for the formation of ND10. Are there any other nuclear functions needed for ND10 formation? Why do so many nuclear proteins meet in this place? (3) ND10 have been shown to be positioned beside SC35; SC35 is also related to transcribed RNA. What is the functional connection between ND10 and RNA? and (4) HIV DNA locates at SC35, not at ND10. HIV DNA is replicated and not integrated DNA (leftover). Given the fact that ND10 are located next to SC35, is it possible that they have any role with regard to HIV DNA?

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We apologize to those friends and colleagues whose pri-

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ORIGINAL ARTICLE

Searching for nuclear export elements in hepatitis D virus RNA

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Abstract

AIM: To search for the presence of cis elements in hepatitis D virus (HDV) genomic and antigenomic RNA capable of promoting nuclear export.

METHODS: We made use of a well characterized chloramphenicol acetyl-transferase reporter system based on plasmid pDM138. Twenty cDNA fragments corresponding to different HDV genomic and antigenomic RNA sequences were inserted in plasmid pDM138, and used in transfection experiments in Huh7 cells. The relative amounts of HDV RNA in nuclear and cytoplasmic fractions were then determined by real-time polymerase chain reaction and Northern blotting. The secondary structure of the RNA sequences that displayed nuclear export ability was further predicted using a web interface. Finally, the sensitivity to leptomycin B was assessed in order to investigate possible cellular pathways involved in HDV RNA nuclear export.

RESULTS: Analysis of genomic RNA sequences did not allow identifying an unequivocal nuclear export element. However, two regions were found to promote the export of reporter mRNAs with efficiency higher than the negative controls albeit lower than the positive control. These regions correspond to nucleotides 266-489 and 584-920, respectively. In addition, when analyzing antigenomic RNA sequences a nuclear export element was found in positions 214-417. Export mediated by the nuclear export element of HDV antigenomic RNA is sensitive to leptomycin B suggesting a possible role of CRM1 in this transport pathway.

CONCLUSION: A cis-acting nuclear export element is present in nucleotides 214-417 of HDV antigenomic RNA.

Key words: Hepatitis D virus; Genomic RNA; Antigenomic RNA; Nuclear export; Nuclear export element

Core tip: Hepatitis D virus (HDV) replicates in the nucleus and export of HDV RNPs to the cytoplasm is thought to be mediated by cis-elements present in virus RNA. We used a chloramphenicol acetyl-transferase reporter system in an attempt to identify the RNA sequences that mediate export to the cytoplasm. Several cDNA constructs coding for different HDV RNA (genomic and antigenomic) sequences were tested. Our results show that a cis-acting nuclear export element is present in positions 214-417 of antigenomic RNA. Two regions in genomic RNA were found to promote nuclear export with efficiency higher than the negative control although lower that the positive control.

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INTRODUCTION

Hepatitis D virus (HDV) is the only member of the Deltavirus genus and is considered to be a satellite virus of the hepatitis B virus (HBV)[1]. When compared with HBV alone, infection of human hepatocytes with both viruses increases liver damage and the risk of cirrhosis and fulminant disease[2,3]. The two viruses are associated due to the fact that the outer envelope of HDV consists of HBV surface antigens (HBsAgs) which are necessary for virus packaging and propagation of infection[4].

The HDV genome consists of a circular, closed ssRNA molecule of approximately 1.7 kb and negative polarity. It is estimated that about 70% of this RNA molecule is internally base-paired resulting in the formation of a rod-like structure similar to plant viroids[5]. There is still a considerable lack of information, and even some controversy, concerning the mechanisms and host factors involved in HDV RNA replication. It seems to be generally accepted that replication occurs through a double rolling-circle mechanism involving the participation of at least host RNA polymerase Ⅱ, and resulting in the synthesis of multimeric antigenomic molecules[6,7]. Subsequently, these multimeric antigenomic molecules are self-cleaved and ligated at precise monomeric intervals by the HDV RNA ribozyme activity[8]. The monomeric antigenomes serve as templates for a second round of replication, by a similar mechanism, thus resulting in the synthesis of monomeric genomic RNA molecules. The HDV genome contains a single ORF that codes for a 24 kDa protein, the so-called small delta antigen (S-HDAg)[9]. As a consequence of an editing mechanism that converts an amber stop codon UAG into a tryptophan codon UGG in the antigenome, the ORF is extended by 19 additional aminoacids[10]. As a result, a 27 kDa protein, the large delta antigen (L-HDAg) is produced. These two proteins are thought to play different roles in the HDV replication cycle. S-HDAg is necessary for accumulation of virus RNA[11] and positively regulates ribozyme activity[12], and L-HDAg inhibits replication and interacts with HBsAgs to promote virus packaging[13,14]. HDV packaging occurs in the cytoplasm where the newly synthesized RNPs meet the HBsAgs to assemble mature virions. It has been previously shown that HDV RNPs shuttle continuously between the nucleus and the cytoplasm[15]. While nuclear import of virus RNPs is mediated by a nuclear localization signal in HDAgs[16], the export to the cytoplasm is believed to be mediated by a cis element present in the RNA molecule. This is supported by the fact that export of both genomic and antigenomic HDV RNAs was found to be independent of the presence of HDAgs[15]. Furthermore, Macnaughton and Lai reported that both genomic and antigenomic RNAs (gRNA and agRNA, respectively) are exported with similar efficiency at early times during replication[17]. Although cells expressing L-HDAg, HBsAgs, and agRNA were found to secret virus-like particles containing HDV agRNA[18], it is widely accepted that only gRNA molecules are packaged into newly synthesized virions. This observation led to the hypothesis that packaging is restricted to gRNA molecules due to the nuclear retention, and eventual further degradation, of HDV antigenomes. However, to our knowledge, no experimental evidences were obtained supporting this idea.

Simple retroviruses such as simian type D retroviruses have evolved mechanisms of RNA export based on the direct interaction of a cis-acting transport element [constitutive transport element (CTE)] with cellular transport receptors. The TAP protein, the human homologue of yeast Mex67p, is one of best studied host factors shown to interact with the CTE to promote nuclear export of unspliced simian retrovirus type D mRNAs[19]. TAP was also identified as one of the proteins responsible for export of cellular mRNAs[20]. On the other hand, complex retroviruses were shown to use a different pathway for export of intron-containing mRNAs. This pathway involves the participation of the cellular protein CRM1[21]. In the case of human immunodeficiency virus-1 (HIV-1), the association of CRM1 with intron-containing mRNAs is mediated by the virus protein Rev which recognizes a specific sequence named rev-responsive element (RRE)[22]. Additionally, the HBV posttranscriptional regulatory element (PRE), which was reported to play a crucial role in export of virus mRNAs to the cytoplasm, seems to use a distinct, not yet identified nuclear export pathway[23].

In an attempt to clarify whether HDV gRNA and agRNA contain cis elements capable of promoting the export to the cytoplasm, we made use of a chloranphenicol acetyl-transferase (CAT) reporter system, in transfection experiments, to identify and characterize putative nuclear export elements.

MATERIALS AND METHODS

Cell culture and transfection

HuH-7 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10%FBS (Invitrogen). Cells were grown as monolayers at 37 ℃, in a humidified atmosphere containing 5%CO_2. Transfection assays were performed using the Fugene6 Transfection Reagent (Roche) and 1 μg plasmid DNA per 35-mm well, according to the manufacturer's instructions. To control for transfection effiency in CAT assays, 20 ng of plasmid pSV-β-galactosidase (Promega) were cotransfected with 100 ng of reporter CAT constructs, and 1.88 μg pUC19. Cells were analysed 24 h post-transfection. In some experiments, 10 nmol/L leptomycin B was added to the medium 18 h after transfection and cells were subsequently incubated for 6 h before analysis as earlier described[24].

Plasmid constructs

Plasmid pDM138[25,26] was a kind gift of Tristram Parslow (Emory University School of Medicine, Atlanta, United States). Plasmids pDM138-PRE(+) and pDM138-PRE(-) were generously provided by Benedict Yen (University of California, San Francisco, United States). Plasmids pDM138-PRE(+) and pDM138-PRE(-) contain a DNA fragment of approximately 570 bp that codes for the

Table 1 Primers used to amplify hepatitis delta virus cDNA fragments

HDV cDNA fragments	*Forward primer* 5'→3'	*Reverse primer* 5'→3'	Fragment length (bp)	Genome location (Sense)	Antigenome location (Antisense)
A1	CGCATCGATACTCCCTGCAGATTGGGGA	CGCATCGATATTCACCGACAAGGAGAGGC	243	360-602	1078-1320
A2	CGCATCGATGCCTCTCCTTGTCGGTGAAT	CGCATCGATGAGACCTCCGGAAGACAAAGA	204	176-379	1301-1504
A3	CGCATCGATTTCCGGAGGTCTCTCTCGAGT	CGCATCGATTCTCCTCGCTCGGAACTTG	214	1654-679/188	1492-1679/26
A4	CGCATCGATTTCCTCGGTCAACCTCCTGA	CGCATCGATATAAGGATGGAGAGGGGGCT	224	266-489	1191-1414
A5	CGCATCGATCCCCTCTCCATCCTTATCCT	CGCATCGATAGGGAGAGAAGAGATCCTCGA	172	114-285	1395-1566
A6	CGCATCGATCGAATGGGACCCACAAATCT	CGCATCGATTCCCCAATCTGCAGGGAGT	337	584-920	760-1096
A7	CGCATCGATCCCAATCCCAGATCTGGAGA	CGCATCGATTTTGCTTTCCTCCTCGCTTC	204	1263-1466	214-417
A8	CGCATCGATAAAGAAAGCAACGGGGCTAG	CGCATCGATGGGAGTCGGAATCGAGCAT	199	1067-1265	415-613
A9	CGCATCGATATGCTCGATTCCGACTCCC	CGCATCGATCCTAGAGAGATTTGTGGGTCCC	191	895-1085	595-785
A10	CGCATCGATCAAGTTCCGAGCGAGGAGAC	CGCATCGATTCTCCAGATCTGGGATTGGG	227	1446-1672	8-234

Nucleotide sequence of the primers used in PCR reactions to amplify hepatitis delta virus (HDV) cDNA fragments. The *Cla* I restriction site is underlined. The size of the amplicon and the corresponding location in the genome, when cloned in sense and antisense orientations, are indicated.

Table 2 Primers used to amplify truncated forms of the A7AS agRNA sequence

HDV cDNA fragments	*Forward primer* 5'→3'	*Reverse primer* 5'→3'	Fragment length (bp)
269-417	CGCATCGATGGGAGGAATCCACTCGGAGA	CGCATCGATTTTGCTTTCCTCCTCGCTTC	149
214-379	CGCATCGATCCCAATCCCAGATCTGGAGA	CGCATCGATGCATCTCCTCCTATCGCTATGG	166
214-403	CGCATCGATCCCAATCCCAGATCTGGAGA	CGCATCGATGCTTCGGTCTCCCCCTACTC	190
244-417	CGCATCGATCCCGAAGGGTTGAGTAGCAC	CGCATCGATTTTGCTTTCCTCCTCGCTTC	174
244-403	CGCATCGATCCCGAAGGGTTGAGTAGCAC	CGCATCGATGCTTCGGTCTCCCCCTACTC	160
314-417	CGCATCGATACCCCTTCAGCGAACAAGAG	CGCATCGATTTTGCTTTCCTCCTCGCTTC	104
214-322	CGCATCGATCCCAATCCCAGATCTGGAGA	CGCATCGATTGAAGGGGTCCTCGGAGGT	109
269-379	CGCATCGATGGGAGGAATCCACTCGGAGA	CGCATCGATGCATCTCCTCCTATCGCTATGG	111

Nucleotide sequence of the primers used to amplify eight truncated forms of the A7AS sequence localized in position 214-417 in the hepatitis delta virus (HDV) antigenomic cDNA. The underlined sequence corresponds to a *Cla* I restriction site.

HBV post-transcriptional regulatory element (PRE). This fragment was inserted in the unique *Cla* I site of pDM138 in both sense and antisense orientations, originating plasmids pDM138-PRE(+) and pDM138-PRE(-), respectively[27].

Twenty vectors containing cDNA inserts corresponding to 10 different regions of the HDV agRNA cloned, in both orientations, in the unique *Cla* I site of pDM138 were generated by polymerase chain reaction (PCR), using the primers listed in Table 1, and plasmid pDL481[18] as template. This plasmid was designed to code for full-length HDV antigenomic RNA and was a kind gift of John Taylor (Fox Chase Cancer Center, Philadelphia, United States). The primers were designed in order to include a *Cla* I site in the 5' end. The obtained PCR fragments were purified using the GFX PCR and Gel Band kit (GE Healthcare) and ligated with *Cla* I digested pDM138 using the Rapid DNA Ligation kit (Roche) according with the instructions of the manufacturer. The correct insertion in sense or antisense orientations of the fragments was first monitored by restriction endonuclease analysis with *Ban* Ⅱ, *Eco*R Ⅰ, *Ban* Ⅱ and *Nhe* Ⅰ, *Pst* Ⅰ, *Xho* Ⅰ, *Bgl* Ⅱ, and *Bam*HI (Fermentas) followed by DNA sequencing.

Additionally, we constructed eight pDM138 derived vectors containing different portions of the cDNA complementary to the HDV agRNA sequence comprised between nt 214 and 417. The strategy was similar to the one described above and the primers used in PCR reactions are listed in Table 2.

Plasmid pDL481ΔNEE, containing full-length HDV agRNA from which the sequence corresponding to the putative nuclear export element (NEE) was removed (nt 2473-2696), was constructed as follows: first we digested plasmid pDL481 with *Apa* Ⅰ (Invitrogen) which cuts at positions 2696 and 3208. The two resulting 5762 bp and 512 bp fragments were separated by electrophoresis, and the 5762 bp fragment was recovered and purified using the GFX PCR DNA kit (GE Healthcare). Subsequently, this fragment was further digested with *Nhe* Ⅰ (GE Healthcare). Two fragments were obtained with 5539 bp and 223 bp, respectively. The 5539 bp fragment was purified as above. The next step consisted of the amplification of the 2696-3208 nt region of plasmid pDL481. To do this, we used the following primers: Fwd 5'GGGCCCGCTTAGCGCCCCTTTTTCTTCCACCTT 3' in which a *Apa* Ⅰ and a *Nhe* Ⅰ restriction sites were included in the 5' end, and Rev 5' GGGCCCACCG-GTGCCCCCTCTCCATCCTTAT 3' in which a *Apa* Ⅰ (underlined) and a *Age* Ⅰ (grey box) restriction sites were also added at the 5' end. The amplified 512 bp fragment was purified as above, and the two 512 bp and 5539 bp

fragments were then ligated using the Rapid DNA Ligation kit (Roche) according to the specifications of the manufacturer. The correct construction of the recombinant vector was tested by digestion with *Xho* I followed by DNA sequencing.

Plasmid pDL481Δδ was constructed by removing a 189 bp sequence, comprised between nucleotides 3208 and 3397 in pDL481. This sequence is complementary to the putative NEE in the HDV antigenome. To do this we first digested plasmid pDL481 with *Bpi* I (Fermentas) which cuts at positions 3098 and 3397 generating two 5975 bp and 299 bp fragments, respectively. Next, the two fragments were purified and incubated with 5 U Klenow enzyme (Fermentas), 0.05 mmol/L dNTPs, and Klenow buffer (Fermentas), for 10 min at 37 ℃ to generate blunt ends. The resulting blunt-ended fragments were digested with *Apa* I (Fermentas) and 4 fragments were obtained with 110, 189, 402 and 5573 bp, respectively. The 5573 bp fragment was purified as above and used in ligation reactions with the 512 bp fragment of pDL481 (nt 2696-3208) which was amplified by PCR as described before. Prior to ligation, compatible ends were generated in the 512 bp amplified DNA fragment. To do this, we first digested this fragment with *Age* I . Following incubation with Klenow and dNTPs to generate blunt ends, as above described, this fragment was next digested with *Apa* I . After digestion, the DNA was purified using the GFX PCR DNA kit (GE Healthcare), and subsequently used in ligation reactions with the 5573 bp fragment. Ligations were performed using the Rapid DNA Ligation kit (Roche) following the instructions of the manufacturer. The correct construction of the recombinant vector was monitored by restriction endonuclease analysis with *Xho* I followed by DNA sequencing.

Plasmid pDL481ΔNEEδ, from which the putative NEE and the corresponding complementary sequence in the HDV antigenome were deleted, was generated by removing a 223 bp sequence between positions 2473 and 2696 and a 189 bp sequence comprised between nucleotides 3208 and 3397 in plasmid pDL481. The first approach consisted of digesting plasmid pDL481 with *Bpi* I followed by generation of blunt ends with Klenow enzyme, as above described for plasmid pDL481Δδ. Next, we digested the two resulting fragments with *Nhe* I . Three fragments of 299, 625 and 5350 bp, respectively, were obtained and separated by agarose gel electrophoresis. The 5350 bp fragment was purified from the gel using the GFX PCR DNA kit (GE Healthcare) and used in subsequent ligation reactions. Before ligation with the 512 bp fragment of pDL481 (nt 2696-3208) obtained by PCR, compatible ends were generated. This was performed by digesting the 512 bp fragment with *Age* I . After filling the resulting cohesive ends with dNTPs, as above described, the obtained blunt fragment was further digested with *Nhe* I . After purification, this DNA fragment was finally ligated with the previously obtained 5350 bp DNA fragment, as described. Finally, we tested the correct construction of the recombinant plasmid by digestion with *Xho* I followed by DNA sequencing.

In situ hybridization

In situ hybridization was performed on pDL481 and pDL542 transfected HuH-7 cells essentially as described[15]. After transfection, cells were incubated at 37 ℃ for 24 h. All fixation, permeabilization, and denaturation steps were exactly as described[15]. Plasmid pSVL(D3) was labeled by nick-translation with digoxigenin-11-dUTP and used as a probe. This plasmid contains a trimer of full-length HDV cDNA cloned in pSVL (GE Healthcare). Hybridization was performed overnight at 37 ℃ and the probe was detected using a monoclonal anti-digoxigenin antibody conjugated with FITC (Roche) and a secondary anti-FITC antibody conjugated with Alexa-488 (Jackson ImmunoResearch Laboratories). Samples were analyzed under a Zeiss META LSM 510 microscope calibrated with multicolor fluorescent beads (Molecular probes). Green fluorescence was detected using a 488 nm Argon laser.

Northern blotting

For Northern blotting, cytoplasmic mRNA was extracted from HuH-7 cells using the Oligotex Direct mRNA Mini kit (Qiagen). For each obtained sample, 10 μg mRNA was separated by formaldehyde agarose gel electrophoresis and transferred to Nylon membranes (Hybond-N, GE Healthcare) using standard protocols[28]. Hybridization was performed using a digoxigenin-11-dUTP (dig-11-dUTP) labeled DNA probe. Plasmid pDM138 was used as template to amplify and label, by asymmetric PCR, a 481 bp region in the ORF of the CAT protein (nucleotide position 109-590). The primers used in PCR reactions were: Fwd 5' GTTCAGCTGGATATTACGGCC 3' and Rev 5' TCACAGACGGCATGATGAAC 3'. Typically, reaction mixtures contained 2 mmol/L $MgCl_2$, 0.2 mmol/L dATP, dCTP and dGTP, 0.13 mmol/L dTTP, 0.07 mmol/L dig-11-dUTP (Roche), 0.1 μmol/L forward primer, 1 μmol/L reverse primer, 10 ng template DNA, 2.5 U Taq DNA polymerase (Fermentas), in PCR buffer for a final volume of 50 μL. After amplification and labeling, probes were purified using the GFX PCR DNA kit (GE Healthcare), and used for hybridization.

Hybridization was performed according to standard protocols[28] and the hybridized probe was detected with a monoclonal anti-digoxigenin antibody conjugated with peroxidase (Roche). Membrane development was achieved with the Lumi-light^{PLUS} Western Blotting Kit, Mouse/Rabbit (Roche) under the conditions indicated by the manufacturer.

Real-time PCR

Nuclear and cytoplasmic HuH-7 cell fractions were obtained according to a previously described method[29], and used for isolation of RNA with the NucleoSpin® RNA/protein kit (Macherey-Nagel) following the manufacturer's specifications. The RNA samples were then treated with DNase I using the DNA-free™ kit (Ambion), also ac-

cording to the instructions of the manufacturer, and used as templates for synthesis of cDNA. cDNA synthesis reactions typically contained approximately 5 μg total RNA, 0.2 μg random primers, 2 mmol/L dNTPs, 200 U Revert Aid™ M-MuLV Reverse Transcriptase (Fermentas), and 20 U RNase inhibitor (Fermentas) in a final volume of 20 μL. Reactions were performed at 42 ℃, for 1 h, and the obtained cDNA was finally purified using the GFX PCR DNA and Gel Band purification kit (GE Healthcare).

Real-time PCR experiments were performed essentially as described[30]. The qPCR Core kit for SYBR® Green I (Eurogentec) was used following the specifications of the manufacturer. Reaction mixtures typically contained 3.5 mmol/L $MgCl_2$, 200 μmol/L each dNTP, 300 nmol/L each primer, 0.025 U/μL HotGoldStar enzyme, and reaction buffer in a final volume of 20 μL. Reactions were performed in 96-well plates with optical caps in a GeneAmp® 5700 Sequence Detector System (all from Applied Biosystems). The PCR program used for amplification was: 10 min at 95 ℃, 40 cycles with 15 s at 95 ℃ and 1 min at 60 ℃. Each sample was assayed in triplicate and analysed with the GeneAmp® 5700 SDS v1.1 software and Microsoft Excel.

The relative quantification of RNA was performed according to the $2^{-\Delta\Delta Ct}$ method earlier described[31]. The β-2-microglobulin gene (*β2MG*; GenBank accession number NM_004048) was used as reference gene to which all the samples were compared with. The program Primer Express™ (Applied Biosystems) and the bioinformatics tool Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) were used to design primers for the reference gene and target HDV cDNA sequence (GenBank accession number M21012). Melting temperature, GC content, length, and secondary structure were taken in consideration for primer design. The cDNA sequences were obtained from GenBank database from NCBI. The primers used in these experiments were, respectively: HDV Fwd 5' CAGAGATTCTCCGGCGTTGT 3', Rev 5' CGGTAAAGAGCATTGGAACG 3'; β2MG Fwd 5' GGCTATCCAGCGTACTCCAA 3', Rev 5' TCACACGGCAGGCATACTC 3'.

Western blotting

For western blot, protein extracts were prepared using with the NucleoSpin® RNA/protein kit (Macherey-Nagel) according to the manufacturer's instructions and dissolved in sample buffer. Proteins were separated by electrophoresis on 12%SDS-polyacrylamide gels, and subsequently electroblotted onto nitrocellulose membranes (Schleicher and Schuell) as previously described[32]. Membranes were blocked with 5% low fat milk powder in PBS, and incubated for 1 h with 1 μg/mL of a primary mouse monoclonal antibody anti-GAPDH (Ambion). After washing with 2% low fat milk powder in PBS, membranes were further incubated with a secondary anti-mouse IgG antibody conjugated with horseradish peroxidase (BioRad). After washing, membranes were rinsed with PBS and subsequently developed using the ECL™ Western blotting analysis system (GE Healthcare).

CAT assay

Determination of CAT expression was performed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Roche). Briefly, 24 h post-transfection HuH-7 cells were washed with ice-cold PBS and incubated with 500 μL lysis buffer for 30 min, at room temperature. After centrifugation, the supernatants were collected, and 200 μL were added to individual wells of the ELISA plate. CAT detection was performed with a polyclonal anti-CAT antibody conjugated with digoxigenin followed by incubation with a monoclonal anti-digoxigenin antibody conjugated with peroxidase (Roche), as indicated by the manufacturer. The concentration of unknown samples was determined from a standard curve constructed from 1:2 serial dilutions of the standards.

To normalize for transfection efficiencies HuH-7 cells were cotransfected with plasmid pSV-β-galactosidase (Promega) and β-galactosidase (β-Gal) expression was monitored using a commercial ELISA kit (Roche). Briefly, 200 μL of the same cell lysis supernatants obtained as above described were loaded onto individual wells of the ELISA plate. The ELISA assay was performed according to the specifications of the manufacturer and β-Gal concentrations were determined from a standard curve obtained from 1:2 serial dilutions of the standards. All assays were performed in triplicate.

RESULTS

HDV gRNA and agRNA are exported to the cytoplasm

It was earlier reported that both HDV gRNA and agRNA are exported to the cytoplasm in HuH-7 cells[15,17]. This export is independent of the presence of HDAgs, and thus it is likely to rely on the direct interaction of the virus RNA with host factors[15]. Furthermore, Northern blot analysis of HuH-7 transfected cells seemed to indicate that the relative amounts of gRNA and agRNA in the nucleus and cytoplasm remained nearly equimolar up to 28 h after transfection[17].

In order to confirm that HDV agRNA is efficiently exported to the cytoplasm of HuH-7 cells we made use of plasmid pDL481[18], which codes exclusively for HDV agRNA, in transfection experiments. Plasmid pDL542[18], which codes exclusively for gRNA, was used in parallel experiments. Preliminary *in situ* hybridization analysis confirmed that both HDV gRNA and agRNA can be detected in the nuclear and cytoplasmic compartments of HuH-7 cells (Figure 1) 24 h post-transfection.

Since this approach did not allow us to determine if gRNA and agRNA are exported to the cytoplasm with similar efficiency with decided to quantify, by Real-time PCR, the amounts of both molecules in the nuclear and cytoplasmic compartments. To do this, HuH-7 cells were transfected with plasmids pDL481 and pDL542 respectively. After 24 h incubation, RNA samples were obtained

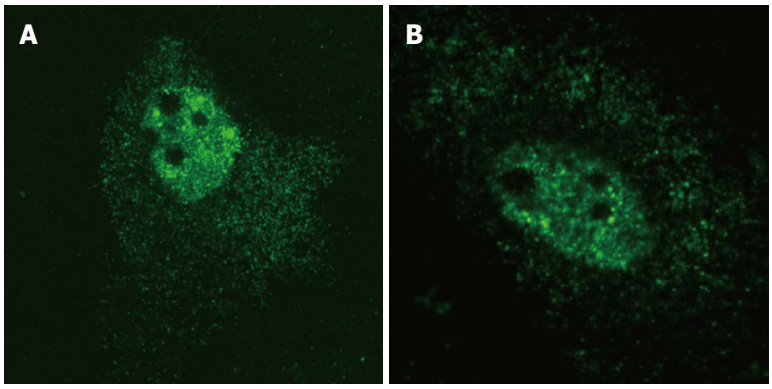

Figure 1 Intracellular localization of hepatitis delta virus gRNA (A) and agRNA (B). HuH-7 cells were transfected with plasmids pDL542 and pDL481, respectively, and virus RNA was detected by *in situ* hybridization with a dig-11-dUTP labeled probe. Both gRNA and agRNA can be observed in the nucleus and cytoplasm (green).

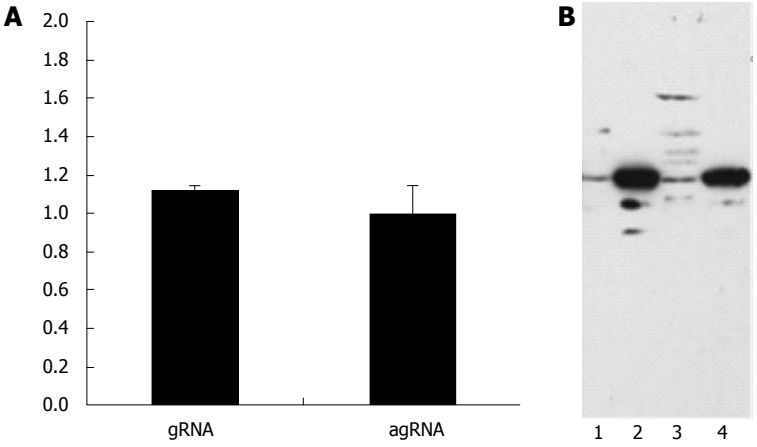

Figure 2 Nucleo-cytoplasmic distribution of hepatitis delta virus gRNA and agRNA. HuH-7 cells were trasnfected with plasmids pDL542 and pDL481, respectively. A: The relative quantification of HDV RNA was performed by real time-polymerase chain reaction using the $2^{-\Delta\Delta Ct}$ method. Results are presented as the cytoplasmic /nuclear ratio (C/N) and correspond to the mean of three independent experiments. Bars indicate the standard deviation; B: Western blotting analysis of nuclear and cytoplasmic HuH-7 cell protein fractions. Equivalent amounts of nuclear (lanes 1 and 3) and cytoplasmic (lanes 2 and 4) protein fractions used for quantification of gRNA (lanes 1 and 2) and agRNA (lanes 3 and 4) were separated in 12% SDS-PAGE gels. The possible contamination of nuclear fractions was monitored by using an anti-GAPDH antibody.

from nuclear and cytoplasmic fractions for subsequent use in Real-time qPCR experiments. The possible cross contamination of nuclear and cytoplasmic fractions was monitored by Western blotting using an anti-GADPH antibody (Figure 2B). The reference gene in qPCR experiments was β-2-microglobulin (Genbank accession number P61769).The obtained results are displayed, as the cytoplasm to nuclear ratio of gRNA and agRNA, respectively, in Figure 2A. The HDV agRNA was found to be distributed in equimolar amounts between the nucleus and cytoplasm of HuH-7 cells 24 h after transfection. At the same time point the HDV gRNA was found in slightly higher amounts in the cytoplasm. These results are consistent with previously reported data and clearly indicate that the virus agRNA is efficiently exported to the cytoplasm.

Identifying nuclear export elements in HDV genomic and antigenomic RNA

After establishing that HDV gRNA and agRNA are exported to the cytoplasm, in the absence of HDAgs, with similar efficiency, until at least 24 h after transfection, we decided to investigate the eventual presence of a cis-acting nuclear export element in both RNA molecules. To do this, we made use of plasmid pDM138[25,26]. This plasmid codes for the second half of the HIV-1/SF2 genome under the control of the SV40 promoter. The DNA sequence coding for the CAT gene was inserted in the HIV-1 envelope gene intron, and the RRE was substituted by a linker containing a unique *Cla* I restriction site. Nuclear export of mRNAs derived from pDM138, and subsequent expression of the reporter CAT protein, is thus dependent on the insertion of a functional transport element in the *Cla* I restriction site.

Initially, we amplified by PCR 20 cDNA fragments covering the entire HDV genome (10 fragments) and antigenome (also 10 fragments). The size of the obtained fragments ranged from 167 to 337 nt and the respective location in the genome is displayed in Table 1. Each fragment was subsequently cloned in the unique *Cla* I site of plasmid pDM138 in both sense and antisense orientations. We thus obtained 20 different constructs which, after being sequenced to confirm the correct insertion and orientation, were used to transfect HuH-7 cells. As positive and negative controls in these experiments we used plasmids pDM138-PRE(+) and pDM138-PRE(-), respectively. These plasmids contain the HBV post-transcriptional regulatory element, cloned in sense and antisense orientations, respectively[27]. In all experiments, plasmid pSV-β-Gal (Promega) was used to cotransfect HuH-7 cells in order to normalize for transfection efficiencies. Twenty four hours after transfection, total protein extracts were prepared and the production of CAT and β-Gal was determined by ELISA. Figure 3 displays the obtained results.

When analyzing gRNA all the tested sequences were found to be unable to promote the export of CAT mRNAs with efficiency as high as that determined for the

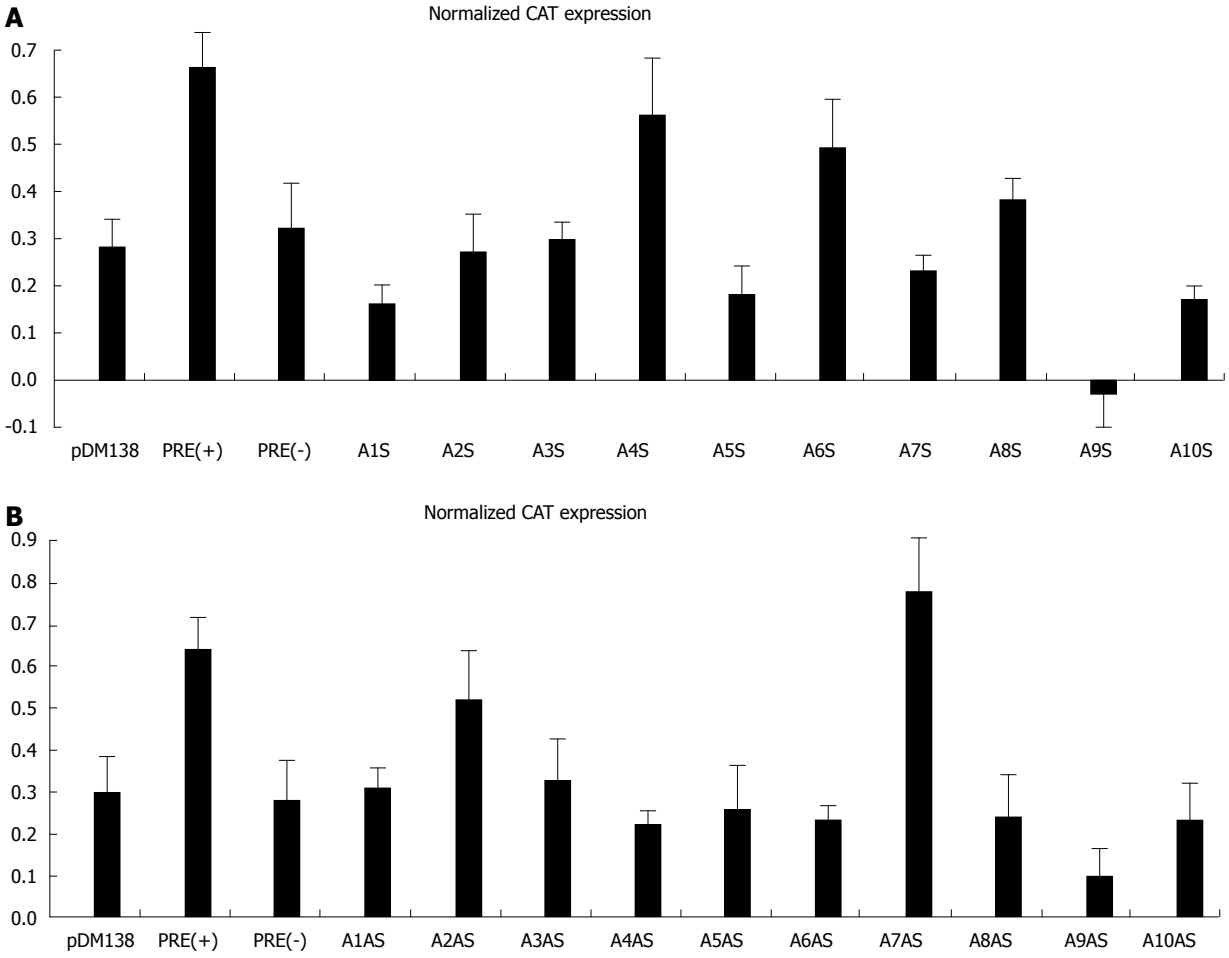

Figure 3 Analysis of chloranphenicol acetyl-transferase expression in HuH-7 cells transfected with plasmids pDM138, pDM138 (PRE+), pDM138 (PRE-), pDM138 A1S-pDM138 A10S (A), and pDM138 A1AS-pDM138 A10AS (B). In order to normalize for transfection efficiency, cells were co-transfected with plasmid pSV-β-Gal (Promega). Chloranphenicol acetyl-transferase (CAT) and β-Gal expression levels were determined by ELISA. Normalization of CAT expression levels was calculated by dividing the values obtained for the CAT protein by the values obtained for the β-Gal protein. The results correspond to the mean of three independent experiments. Bars represent the standard deviation.

positive pDM138-PRE(+) control. Nevertheless, two of the analyzed gRNA sequences, corresponding to nucleotides 266-489 and 584-920 (A4S, and A6S, respectively), showed an export-promoting ability slightly lower than the positive control albeit clearly higher than the negative control pDM138-PRE(-).

The analysis of agRNA coding sequences, however, showed that in HuH-7 cells transfected with plasmid pDM138-A7AS, which contains the HDV agRNA sequence corresponding to nucleotides 214-417, the expression levels of the reporter CAT protein are higher than those detected for the pDM138-PRE(+) positive control.

With the exception of A7AS and A2AS sequences, all the remaining tested constructs were found to be unable to promote the export of heterologous intron-containing mRNAs since the detected CAT expression levels were comparable or even lower than those observed in negative pDM138-PRE(-) transfected HuH-7 cell controls. The CAT expression values obtained for the A2AS sequence, however, were found to be intermediate between those obtained for the positive pDM138-PRE(+) and negative pDM138-PRE(-) controls.

According to what has been previously reported for the HBV PRE[27], the export promoting activity of the identified A7AS sequence in the HDV antigenome is dependent on its orientation relative to the ORF of the reporter gene. In fact, when cloned in opposite orientation (A7S), the A7AS sequence (nt 214-417) was not functional, since the observed CAT expression levels were similar to those found for the pDM138-PRE(-) negative control (data not shown).

Since the quantification of CAT expression levels by ELISA represents an indirect approach for the determination of intron-containing mRNAs accumulation in the cytoplasm, we decided to investigate the presence of CAT mRNAs in cytoplasmic fractions, after transfection, by Northern blot. To do this, HuH-7 cells were transfected with plasmid pDM138-A7AS or plasmids pDM138-PRE(+) pDM138-A9AS as positive and negative controls, respectively, and after 24 h incubation total and cytoplasmic fractions were prepared and used for further mRNA extraction. After electrophoresis, the mRNA samples were transferred to nylon membranes, and a single-stranded dig-11-dUTP labeled DNA probe,

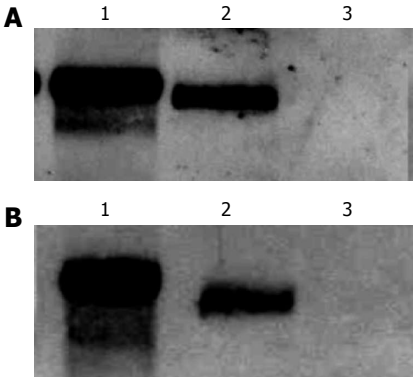

Figure 4 Northern blotting analysis of reporter chloranphenicol acetyltransferase mRNA in total (A) and cytoplasmic (B) fractions of HuH-7 cells transfected with plasmids pDM138 (PRE+), pDM138 A7AS, and pDM138 A9AS (lanes 1, 2 and 3 respectively). Hybridization was performed using a dig-11-dUTP labeled probe. A peroxidase conjugated anti-digoxigenin antibody was used to detect the hybridized probe.

which specifically hybridizes with CAT mRNA, was used in Northern blot assays. As expected, it was possible to detect the presence of CAT mRNA in cytoplasmic fractions of pDM138-PRE(+) and pDM138-A7AS transfected HuH-7 cells but not in pDM138-A9AS transfected cells (Figure 4).

The absence in Northern blot experiments of detected CAT mRNA in total and cytoplasmic fractions of pDM138-A9AS transfected cells is compatible with the data obtained by ELISA. The A9AS sequence includes the agRNA autocatalytic ribozyme domain. It is, thus, possible that CAT mRNAs that include the A9AS sequence are degraded in the nucleus before export to the cytoplasm. In conclusion, these results indicate that the HDV agRNA sequence located between nucleotides 214-417 can efficiently promote the export of heterologous intron-less RNAs. Moreover, the increase in CAT expression levels observed in pDM138-A7AS transfected cells is a consequence of the export and accumulation of the respective reporter mRNAs in the cytoplasm.

Analysis of the structure of the nuclear export element in agRNA

Having established that the HDV agRNA sequence corresponding to nucleotides 214-417 (A7AS) is able to promote the nuclear export of heterologous intron-containing RNAs, we next decided to analyze it in more detail. First, we generated by PCR eight truncated forms of the A7AS motif and cloned them in the unique *Cla* I site of plasmid pDM138. These truncated forms correspond to the A7AS sequence from which several nucleotides were removed from the 5' and 3' ends. The constructs were subsequently used to transfect HuH-7 cells, and after 24 h CAT expression was determined by ELISA. The obtained CAT expression values were normalized for transfection efficiency by cotransfection with plasmid pSV-β-Gal followed by determination of β-Gal expression. The obtained results allowed us to conclude that the agRNA sequences comprised between nucleotides 214-322, 214-379, 214-403, 244-403, and 244-417 induce an increase in CAT expression comparable to the observed for the HBV PRE(+) positive control (Figure 5) suggesting that these sequences are sufficient to promote export of heterologous RNAs.

Additionally, the first 30 nucleotides localized 3' in the A7AS sequence seem not to be crucial to promote nuclear export since its deletion did not significantly affect the detected amounts of CAT expression. All the remaining analyzed sequences, 269-417, 314-417, and 269-379 were found to be considerably less efficient in promoting nuclear export.

Analysis of the NEE function in the context of the full-length agRNA

The identified NEE is localized in the central region of the rod-like full-length agRNA molecule. It could be possible that the complementary RNA sequence that pairs with the NEE in the antigenome is possibly also involved in the nuclear export of the agRNA. To test this hypothesis we constructed three deletion mutants of plasmid pDL481, which codes for the complete HDV agRNA molecule: pDL481ΔNEE in which the NEE was deleted (nt 214-417), pDL481Δδ in which the complementary to the NEE sequence was deleted (nt 1179-1385), and pDL481ΔNEEδ which does not contain the NEE and the corresponding complementary sequence (nt 214-417 and 1179-1385, respectively).

Plasmids pDL542, pDL481, and the obtained deletion constructs were used to transfect HuH-7 cells. Twenty four hours post-transfection, nuclear and cytoplasmic fractions were prepared and the RNAs derived from pDL542, pDL481, pDL481ΔNEE, pDL481Δδ, and pDL481ΔNEEδ, respectively, were quantified by qRT-PCR. Possible cross contaminations of nuclear and cytoplasmic fractions were monitored by western blot using an anti-GAPDH antibody as described before. The obtained results confirmed that both gRNA and agRNA are efficiently exported to the cytoplasm 24 h post-transfection. Additionally, the RNA derived from plasmid pDL481Δδ, in which the sequence coding for the region complementary to the NEE was deleted, showed to be efficiently exported (Figure 6). In contrast, the RNAs coded by plasmids pDL481ΔNEEδ and pDL481ΔNEE were mostly retained in the nucleus. Only about 40% of the total amount of these RNAs was found in the cytoplasm of transfected cells when compared to wt pDL481. These results indicate that the complementary to the NEE sequence in the HDV antigenome (nt 1179-1385) is not involved in nuclear export since its deletion did not reduce the amounts of agRNA detected in the cytoplasm.

HDV agRNA export is sensitive to leptomycin B

Export of cellular RNAs is accomplished using different pathways which involve the participation of distinct transport receptors. Typical examples include the export of mRNAs, which is mediated by members of NXF family of proteins, namely the TAP protein, and the

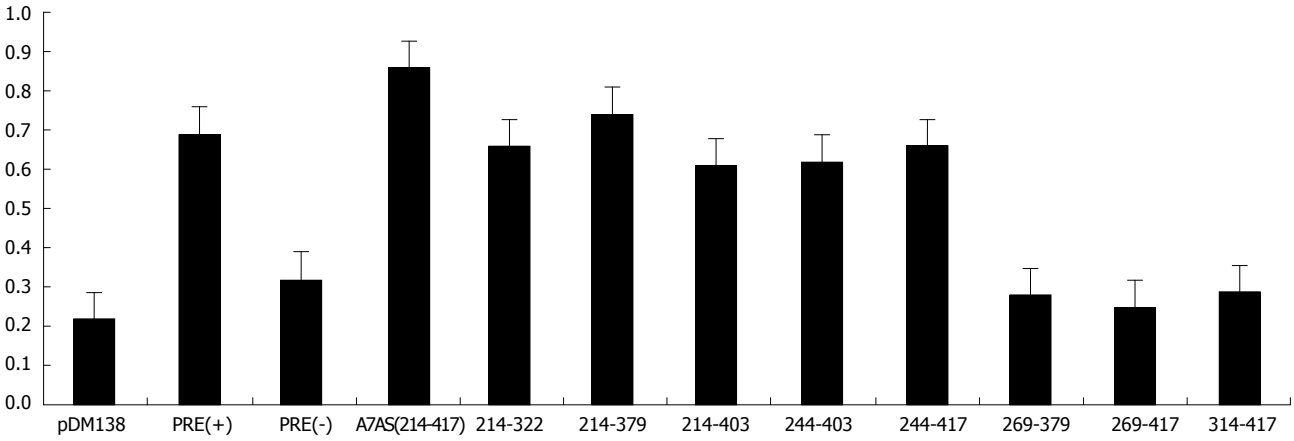

Figure 5 Analysis of chloranphenicol acetyl-transferase expression in HuH-7 cells transfected with plasmids pDM138, pDM138 (PRE+), pDM138 (PRE-), pDM138 A7AS (214-417), pDM138-314-417, pDM138-214-322, pDM138-269-379, pDM138-244-379, pDM138-269-417, pDM138-214-403, pDM138-244-403, and pDM138-244-417. Chloranphenicol acetyl-transferase (CAT) and β-Gal expression levels were determined by enzyme-linked immunosorbent assay. The CAT expression values were normalized for transfection efficiency by transfecting HuH-7 cells with plasmid pSV-β-Gal (Promega) followed by determination of β-Gal expression. CAT expression values were divided by the corresponding β-Gal expression values, and the displayed results correspond to the mean of three independent experiments.

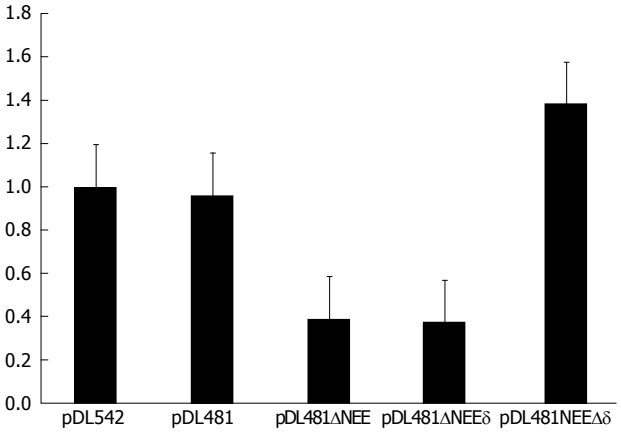

Figure 6 Nucleo-cytoplasmic distribution of hepatitis delta virus RNA in HuH-7 cells transfected with plasmids pDL481, pDl542, pDL481ΔNEE, pDL481ΔNEEδ, and pDL481Δδ. The RNA in nuclear and cytoplasmic cell fractions was determined by real time-polymerase chain reaction using the $2^{-\Delta\Delta Ct}$ method. Results are presented as the cytoplasmic /nuclear ratio (C/N) and correspond to the mean of three independent experiments. Bars indicate the standard deviation.

export of UsnRNAs which is mediated by the exportin CRM1[20]. This specific export pathway can be inhibited in the presence of leptomycin B (LMB)[33]. In order to investigate whether HDV agRNA is exported to the cytoplasm using the pathway mediated by CRM1, we decided to analyze the effect of LMB on CAT protein expression in pDM138-A7AS transfected HuH-7 cells. To do this, 10 nmol/L LMB was added to the culture medium 18 h after transfection, and cells were further incubated for 6 h. Total protein extracts were then prepared and used to determine the concentration of CAT by ELISA. As negative control pDM138-PRE(+) transfected HuH-7 cells were used since it was previously reported that export of HBV PRE(+) is not sensitive to LMB[23,34]. Figure 7 displays the obtained results. As expected, in the absence of LMB both the HDV agRNA A7AS and HBV PRE(+) sequences promote the export of CAT mRNA thus confirming our previous data. In the presence of LMB the export capacity of the HBV PRE(+) sequence is not affected, and we could detect a slight increase in CAT expression. This observation is in accordance with the data obtained by others when measuring CAT enzyme activity in similar experiments[34]. In contrast, the export promoting activity of the HDV agRNA A7AS sequence was found to be affected in the presence of LMB. In fact, we observed a 60% reduction in CAT production when HuH-7 cells were transfected with pDM138-A7AS in the presence of 10 nmol/L LMB. These results seem to indicate that the nuclear export mediated by the A7As agRNA sequence is dependent, at least partially, on CRM1 activity and suggest the involvement of this cellular protein in HDV agRNA export.

DISCUSSION

Although HDV RNA replication occurs in the nucleus of liver cells, virus packaging takes place in the cytoplasm where HDV RNPs meet HBsAgs to assemble newly synthesized virions. Noteworthy, only gRNA molecules were found, until now, to be packaged into mature hepatitis delta virions. This restriction could be due to a possible impairment of export of agRNA molecules to the cytoplasm. However, agRNA was found in HDV virus-like particles secreted by cells expressing agRNA, L-HDAg, and HBsAgs[18].

A previous work showed that HDV RNPs are exported to the cytoplasm independent of the presence of HBsAgs[15]. Additionally, it was demonstrated that export of HDV RNPs is not mediated by a putative nuclear export signal present in delta antigens but is rather promoted by cis elements in virus RNA. In fact, both HDV gRNA and agRNA are exported to the cytoplasm in the absence of

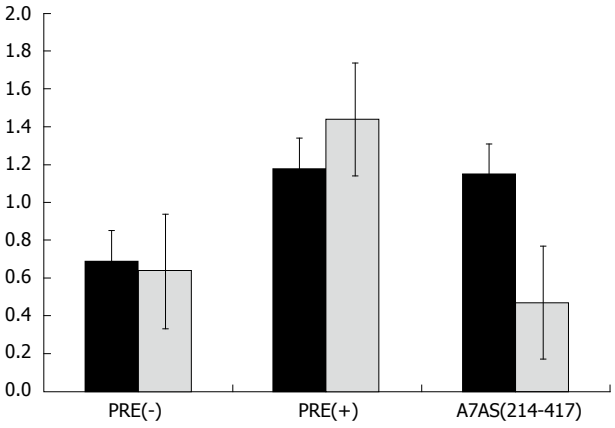

Figure 7 Analysis of chloranphenicol acetyl-transferase expression in HuH-7 cells transfected with plasmids pDM138 (PRE-), pDM138 (PRE+), and pDM138 A7AS, in the absence (black columns) and presence of 10 nmol/L leptomycin B (grey columns). In order to normalize for transfection efficiency, cells were co-transfected with plasmid pSV-β-Gal (Promega). Chloranphenicol acetyl-transferase (CAT) and β-Gal expression levels were determined by enzyme-linked immunosorbent assay. Normalization of CAT expression levels was calculated by dividing the values obtained for the CAT protein by the values obtained for the β-Gal protein. The results correspond to the mean of three independent experiments. Bars represent the standard deviation.

HDAgs, an observation also reported by Macnaughton *et al*[17].

Our first approach was designed to confirm that both HDV genomic and antigenomic RNA molecules are exported to the cytoplasm of HuH-7 cells. Using plasmids pDL542 and pDL481 which express exclusively HDV gRNA and agRNA, respectively, we were able to detect both molecules, by *in situ* hybridization, in the nuclear and cytoplasmic compartments of HuH-7 transfected cells. Furthermore, nuclear export of HDV gRNA and agRNA seems to occur with the same efficiency since similar amounts of genomic and antigenomic RNA molecules could be detected, by real time-PCR, in the nuclear and cytoplasmic compartments of HuH-7 cells transiently transfected with plasmids pDL542 and pDL481. The finding that both genomic and antigenomic HDV RNA molecules are exported with similar efficiency suggests a possible biological function associated with the presence of HDV agRNA in the cytoplasm. However, further research is mandatory to clarify the biological significance of these findings.

The above described observations are suggestive of a possible presence of cis-acting nuclear export elements in HDV RNA, both genomic and antigenomic. In an attempt to identify putative cis elements in the HDV gRNA and agRNA capable of mediating the export to the cytoplasm, we used a well characterized CAT reporter system previously used by others to investigate the role of the HBV PRE in export of intronless mRNAs[27]. This system is based on plasmid pDM138 which was generated in order to contain the second half of HIV-1 cDNA under the control of the SV40 promoter[25,26]. This vector was further engineered in order to remove the initiation codons for the Rev and Env proteins, to include the cDNA sequence coding for the CAT protein, and to substitute the HIV-1 RRE sequence by a linker containing a *Cla* I restriction site. After transcription, an mRNA containing the ORF for the CAT protein inserted in the intron of the HIV-1 Env proteins is produced. As positive and negative controls we used plasmids pDM138-PRE(+) and pDM138-PRE(-), respectively. These plasmids contain the HBV post-transcriptional regulatory element (PRE) inserted in the unique *Cla* I site of pDM138, in sense and antisense orientations, respectively[27]. We cloned several cDNA fragments, covering the entire HDV gRNA and agRNA, in plasmid pDM138. After transfection of HuH-7 cells, CAT production was determined by ELISA. This approach did not allow identifying unequivocally a nuclear export element in gRNA. Although two gRNA fragments corresponding to nucleotides 266-489 and 584-920, (A4S and A6S, respectively) were found to promote export of CAT mRNAs, the efficiency was in all experiments lower than the determined for the positive control pDM138-PRE(+). This could be possibly due to the lack of crucial nucleotides in the ends of the analyzed sequences. Clearly, additional experiments are mandatory to clarify this point.

However, we were able to identify a region (A7AS) in the HDV agRNA, located between positions 214-417, which promoted the export of CAT mRNA with slightly higher efficiency than that observed for the pDM138-PRE(+) positive control. This result was further confirmed by Northern blot analysis of total and cytoplasmic mRNAs prepared from pDM138-PRE(+) and pDM138A7AS transfected HuH-7 cells. Moreover, when inserted in the opposite orientation in pDM138, the fragment A7AS was not able to mediate export of CAT mRNAs (data not shown). All the other fragments tested were not capable of promoting export of CAT mRNA at levels comparable to those observed for the pDM138-PRE(+) positive control. In particular, the fragment A9AS which includes the HDV RNA ribozyme sequence displayed a CAT mRNA export capacity significantly lower than the calculated for the negative control. This may be a consequence of instability of the produced mRNA molecules due to the self-cleavage activity of the HDV ribozyme. Besides the A7AS sequence, the only exception to the low export promoting capacity of the analyzed fragments, concerns a cDNA fragment corresponding to positions 1301-1514 in the antigenome (A2AS). This agRNA fragment was able to promote CAT mRNA export with efficiency higher than the negative control but still lower than the determined for the positive control. This fragment, A2AS, includes part of the HDAg ORF. This observation may allow speculating about a possible presence of a cis element in the HDV mRNA involved in export to the cytoplasm. Huang and Carmichael have previously shown that export on intronless histone H2a mRNAs is mediated by a signal present in the coding region[35]. However, further experiments are needed to clarify the possible involvement of a similar signal in export of HDAg mRNA.

In an attempt to analyze in more detail the A7AS sequence in the HDV agRNA we generated, by PCR, eight truncated forms of this motif which were subsequently cloned in pDM138 and used in transfection experiments to determine CAT expression by ELISA. These truncated forms corresponded to nucleotides 314-417, 269-417, 244-417, 244-403, 214-403, 269-379, 214-379, and 214-322. The obtained results showed that deletion of the first 30 nucleotides in the 5' end of the A7AS sequence did not significantly affect the export promoting capacity. However, deletion of the first 55 nucleotides in the 5' end results in loss of the export capacity of the A7AS sequence. In contrast, deletions in the 3' end of the A7AS motif, did not significantly affect the ability to promote nuclear export. In fact, removal of as much as the first 95 nucleotides in the 3' end still resulted in the production of the CAT protein at intermediate levels between the negative and positive controls. Noteworthy, all the analyzed A7AS truncated forms were less efficient in promoting RNA export when compared to the wild-type sequence.

However, analysis of the secondary structure of the entire agRNA molecule did not allow predicting a similar branched structure in the region where the A7AS motif is located. In order to clarify a possible role of a, at least partially, complementary to the A7AS motif sequence in the antigenome, in nuclear export, we constructed deletion mutants of plasmid pDL481. These constructs were designed as follows: pDL481ΔNEE lacks the nuclear export element (A7AS sequence), pDL481Δδ lacks the complementary to the NEE sequence in the antigenome, and pDL481ΔNEEδ lacks both the NEE and the respective complementary sequence. After transfection of HuH-7 cells, the relative amounts of agRNA in nuclear and cytoplasmic fractions were determined by qRT-PCR. The obtained results showed that deletion of the NEE (pDL481ΔNEE) reduces export of agRNA by 60% when compared with wt pDL481. Additionally, deletion of both the NEE and the respective complementary sequence (pDL481ΔNEEδ) results in a similar reduction (62%) of detected cytoplasmic RNA. Finally, deletion of only the complementary to the NEE sequence did not impair the capacity of the agRNA to be exported to the cytoplasm. Taken together, these results indicate that the identified NEE is important to promote nuclear export of the HDV antigenome, and that export efficiency is not diminished by deletion of the respective putative complementary sequence.

Nuclear export of host and virus RNAs may be promoted by several cellular factors that participate in distinct pathways. One of these pathways is mediated by the exportin CRM1 which belongs to the kariopherin-β family of proteins. CRM1 mediates the export of the majority of proteins containing a nuclear export signal (NES) and of two classes of cellular non-coding RNAS, rRNAs and UsnRNAs[21]. The CRM1 export pathway may be specifically inhibited in the presence of LMB which binds to a cysteine residue in the central region of the protein[33]. In order to investigate a possible involvement of CRM 1 in export of HDV agRNA we used LMB to inhibit this pathway in pDM138-A7AS transfected HuH-7 cells. As positive and negative controls we used plasmids pDM138-PRE(+) and pDM138-PRE(-), respectively, since export of the HBV PRE was previously reported to be insensitive to LMB. The obtained results showed that RNA export mediated by the NEE of HDV agRNA is partially inhibited in the presence of LMB, displaying a 50% reduction of CAT expression when compared with the parental pDM138 vector. This suggests an involvement of the CRM1 protein in export of HDV agRNA. However, since CRM1 binds to RNA molecules indirectly through interaction with other NES-containing proteins this implies the participation of other not yet identified host factors in HDV agRNA export. Not surprisingly, using coimmunoprecipitation assays we couldn't detect complexes between HDV RNPs and CRM1 (our unpublished data).

In this work we attempted to identify nuclear export elements present HDV gRNA and agRNA. Although two regions in gRNA were found to be able to promote export of heterologous RNAs, an unequivocal NEE could not be identified in gRNA. However, it was possible to identify a NEE in HDV agRNA located in positions 214-417. This cis element is not only capable of promoting the nuclear export of heterologous intronless RNAs but is also involved in export of HDV antigenomes. Analysis of the export capacity of several truncated forms of the NEE showed that the two mini-helices seem to play a crucial role in mediating RNA export. Cytoplasmic export of HDV agRNA was found to be sensitive to leptomycin B suggesting a possible involvement of a CRM1 mediated pathway.

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COMMENTS

Background

Hepatitis delta virus (HDV) is a satellite of hepatitis B virus (HBV). The two viruses share the same envelope which consists of HBV surface antigens (HBsAg). HBsAgs are coded exclusively by the HBV genome and are localized in the cytoplasm of infected cells. In contrast HDV replicates in the nucleus and assembly of mature virions takes place in the cytoplasm. Accordingly, HDV RNPs need to be exported to the cytoplasm and this process is thought to be mediated by cis-elements present in the virus RNA.

Research frontiers

Export of RNA molecules to the cytoplasm depends on the interaction of cellular transport receptors with specific nucleotide sequences. These sequences were previously studied in simple and complex retroviruses as well as in HBV. The cellular proteins and pathways involved in virus RNA export were also identi-

fied. Here, the research hotspot is the identification of nucleotide sequences in HDV RNA capable of promoting export of heterologous RNAs and thus being able to function as nuclear export elements.

Innovations and breakthroughs

This is the first report describing a comprehensive analysis of HDV genomic and antigenomic RNA sequences aiming to identify cis-elements capable of promoting export to the cytoplasm. Two possible regions corresponding to nucleotides 266-489 and 584-920, respectively, were identified in HDV genomic RNA. In addition, analysis of HDV antigenomic RNA sequences allowed finding a nuclear export element in positions 214-417. Furthermore, export mediated by the nuclear export element of HDV antigenomic RNA was found to be sensitive to leptomycin B suggesting a possible role of the cellular protein CRM1 in this transport pathway.

Applications

The results herein described may contribute to a better understanding of an essential step in the HDV replication cycle-the export of virus RNPs to the cytoplasm where they interact with HBsAgs in order to assemble new virions.

Terminology

Nuclear export element is an RNA sequence capable to interact with cellular proteins that promote export from the nucleus to the cytoplasm.

Peer review

The paper is of good quality as research article, its english is perfect and its scientific content is original.

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Identification of novel silent HIV propagation routes in Pakistan

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Abstract

Human immunodeficiency virus (HIV) is rapidly increasing in both high risk groups and the general population. In this study, silent routes of propagation in teenaged Pakistanis are discussed. In order to promote sexual activity in youths, regular clients write contact details of sex workers on the doors of public washrooms. HIV prevalence is much higher among Hijra sex workers. Hijra sex workers have apparently stepped into the profession of begging at public places, where they earn money by both begging and distributing visiting cards offering unsafe sex. In many educational institutes, sex education is lacking or absent; if delivered *via* teachers, government agencies and nongovernmental organizations this could prevent a future epidemic of sexually transmitted infections in Pakistan.

Key words: Human immunodeficiency virus; Pakistan; Sex workers; Silent route; Youth

Core tip: Human immunodeficiency virus (HIV) is an emerging threat for rising generations in Pakistan. The prevalence of HIV is gradually increasing in different high risk populations due to the rapid increase of sexual activity among Pakistani youth. Increased sexual desperation among Pakistani youngsters has outranked the rest of the world. The rate of HIV spread in Pakistan is much greater than in any of the western countries, therefore adequate preventive measurements should be implemented as soon as possible, otherwise it will be too late.

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TO THE EDITOR

Human immunodeficiency virus (HIV) is an emerging threat for rising generations in Pakistan. The prevalence of HIV is gradually increasing in different high risk populations due to rapid increase of sexual activity among Pakistani youth. Maximum viral spread and stable maintenance in the environment depends upon effectively penetrating protecting barriers[1]. Prevalence of sexually transmitted diseases was limited to certain areas but unfortunately, now, its prevalence has rapidly increased in different cities. Pakistan is an Islamic republic, where religion, law, culture and society strictly forbid sex outside marriage. Despite the recognition of sex outside marriage as a crime, commercial sex is being offered secretly in various brothels, kothikhana, client homes, rental houses near truck stops and market places, massage parlors and hotels. Strict punishments against sex outside marriage, if implemented by any nation, could prevent individuals from extra marital sexual activity.

We have identified silent routes stimulating sexual

activity among teenaged Pakistanis. Most of the regular clients of sex workers write sexually attractive sentences about female sex workers on the doors of public toilets (in public places such as markets, parks, picnic places, hotels and bus stands) in order to promote sex in the younger generation. Contact details of female sex workers are randomly distributed in public washrooms. Sexually desperate youngsters, introduced to the opportunity, indulge in risky sexual activities which expose them to sexually transmitted infections like HIV and acquired immunodeficiency syndrome. At the moment, fortunately, the prevalence of HIV in the general population is very low. However, if unsafe sexual practices stimulated by this rapid advertisement route, were to be carried out by youngsters then this would probably generate an HIV epidemic in society which could create an extra burden on the economy. Another highly dangerous route of sexual advertisement is through Hijra sex workers. In Pakistan, the prevalence of HIV is highest among injecting drug users (IDUs) and Hijra sex workers. The greatest HIV prevalence (37.8%) was found in IDUs from different cities of Pakistan[2,3]. An extremely high HIV prevalence (27.6%) was observed in Hijra sex workers of Larkana Sindh, Pakistan[4]. Similarly another study conducted in Rawalpindi (the city adjoining Pakistan's capital) reported a high prevalence of HIV (21.6%) among transgender men[5]. The rate of HIV spread in Pakistan is much higher than in any of the western countries, therefore adequate preventive measurements should be introduced as soon as it is possible.

Hijra is a distinct type of gender role which includes Khusras (true hermaphrodites), Zananas (crossdressers) and Narnbans (eunuchs)[6]. We have identified that the majority of Hijra sex workers have started promoting sexual activity in the younger generation by distributing various visiting cards among shop keepers and youth as sexual advertisements. Mobile numbers and visiting addresses are written on those cards. Sexually active people usually make a phone call and arrange a time and place for unsafe sexual activities. Hijra sex workers have also stepped into begging at various public sectors including bus stands, markets and traffic signals, where they not only earn money by begging but also promote sex work by contacting youths. Hijras usually flatter the physical beauty and power of their clients thus promoting sexual relationships. Most of the youngsters who indulge in risky sexual relationships are illiterate and have a poor understanding of sexually transmitted diseases and their consequences. Most of them lack awareness and knowledge, and are therefore unable to comprehend the seriousness of this issue.

In educational institutes such as schools and colleges, sex education is mostly avoided. Teachers are often reluctant to educate students with knowledge about awareness and prevention of sexually transmitted disease. If appropriate seminars were arranged for teenaged students this would be a positive step towards awareness and prevention of HIV and other sexually transmitted diseases. In order to reduce any future epidemic of sexually transmitted infections, harm reduction strategies must be implemented rather than intimidation by law enforcement agencies. The mysterious sex industry is based upon the principle of demand and supply. Sexually desperate unemployed youths, tourists, business men, public officials, migrant workers, truck drivers and traders with conspicuous wealth create a strong demand for sexual services. On the other hand, various social and economic factors like acute poverty, unemployment, limited knowledge and skills and the opportunity of a high income through sex, usually compel poor individuals towards offering sex. In such circumstances, suppressing measures *via* law enforcement agencies could further aggravate the spread of hidden risky behavior in society. It has been observed that Hijra sex workers have a strong wish to earn money by positive means but unfortunately our society does not accept them in either the private or public sector. The majority of Hijra sex workers are unable to earn money by positive means due to problems of sexual harassment from sexually active people. By arranging positive support for sex workers, *via* assistance from government sectors and nongovernmental organizations (NGOs) to earn an honorable livelihood, their risky sexual activities and behaviors can be reduced significantly.

Policy makers should not only provide wider opportunities for the dissemination of awareness and knowledge about risk factors associated with viral transmission among populations at risk; but also focus on identification of epidemiological patterns associated with sexually transmitted infections among various high risk populations in Pakistan. At the moment, an inadequate surveillance system for sexually transmitted infections exists with inadequate identification of subpopulations where deadly pathogens are secretly propagating. There is very little knowledge in the general and high risk populations about access to anti-retroviral therapy clinics. Expanded scientific research is also an important factor for successful surveillance of sexually transmitted infections. Among target populations, simple diagnostic tests against HIV could provide information regarding prevalence and trend of HIV infection. If the serum sub-samples are screened against other sexually transmitted infections (like hepatitis C virus and herpes simplex virus 2) after recording valuable basic demographic and behavioral data, this would help to identify behavior risk trends over time and aid surveillance. Another possible way towards harm reduction in Pakistan is by supporting community organizations and NGOs. The mysteries of complex HIV (and other sexually transmitted infection) dynamics could also be investigated using molecular epidemiology studies and mathematical modeling.

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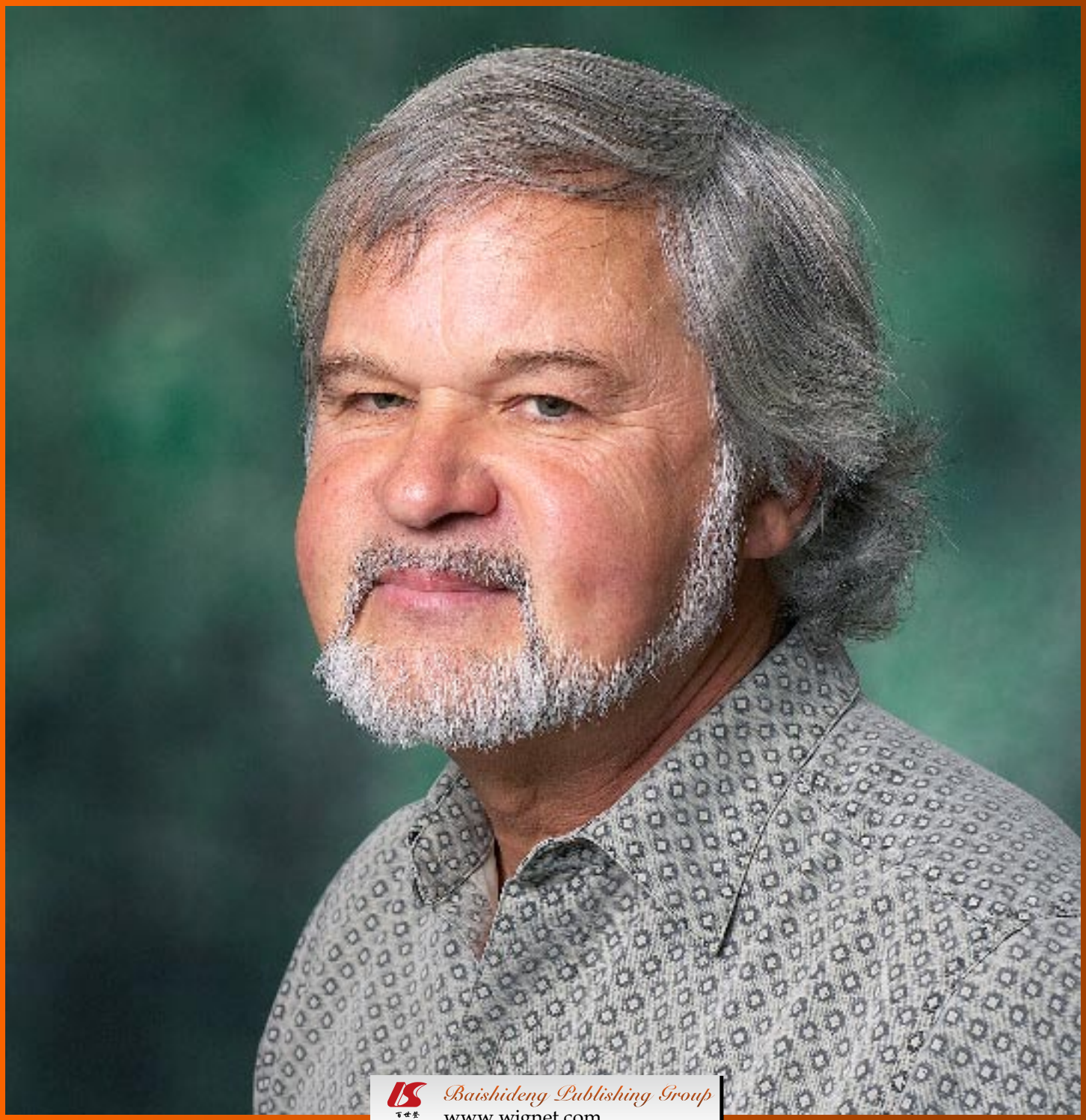

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MINIREVIEWS

Does prophylactic antidepressant treatment boost interferon-alpha treatment completion in HCV?

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Abstract

Depression is often a side effect of interferon-alpha treatment for hepatitis C, and is recognized as a cause for treatment discontinuation. When detected, antidepressant treatment begins promptly. In contrast to this rescue approach, prophylactic antidepressant treatment has been considered as a superior approach. While studies indicate that depression is lower with prophylaxis, no study has prospectively evaluated the degree that treatment completion might be boosted by the prophylactic strategy. A structured literature search was conducted to discover all trials of antidepressant prophylaxis for patients undergoing antiviral treatment for chronic hepatitis C. Selection criteria included: antidepressant prophylaxis study; report of depression treatment outcome; report of numbers discontinuing and reason for discontinuation (including any of the following: discontinuation data for medical side effects (*i.e.*, thrombocytopenia); discontinuation due to lack of antiviral response; discontinuation due to lack of antidepressant effect; discontinuation due to antidepressant side effects; discontinuation due to patient preference; discontinuation due to loss to follow-up; or unspecified discontinuation). Across the studies, total enrollees were determined for the prophylaxis arms and the rescue arms, and then, again across studies, those discontinuing for reasons other than lack of antiviral response or medical side effect were summed for each of these two arms. Twelve studies were discovered. One was a retrospective chart review, one was an uncontrolled trial, and ten were controlled trials. Discontinuation of antiviral therapy was not less common in the prophylaxis arms: of the 396 patients treated by the prophylaxis strategy, 47 (11.9%) discontinued; of the 380 patients in the rescue strategy, 45 (11.8%) discontinued. While the prophylaxis strategy seems to manage depression symptoms, it does not seem to boost treatment completion. Rescue was a very successful strategy when indicated. While antidepressant prophylaxis has benefit in antiviral treatment, it should not generally be valued for boosting the likelihood of treatment completion.

Key words: Depression; Therapy; Clinical; Psychiatry

Core tip: To inform clinical practice, this narrative review summarizes existing evidence regarding the degree that antidepressant prophylaxis boosts hepatitis C antiviral treatment completion compared to a rescue approach.

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INTRODUCTION

Although pegylated interferon-alpha may provide a sustained viral response from chronic hepatitis C infection[1,2],

this lengthy regimen is challenging to tolerate. Depressive symptoms, one of the more difficult side effects, can lead to discontinuation. Discontinuation rates for factors other than antiviral non-response range from 10% in well-conducted clinical trials[1,2] to 30% or more in clinical settings[3]. If depressive symptoms emerge, they must be clinically managed, including suspension of antiviral treatment as a last resort. Direct-acting antiviral agents may eventually supplant interferon-alpha/ribavirin regimens as standard of care[4], but interferon-alpha-based regimens have recently been re-affirmed as standards of care[5,6].

To reduce the threat of treatment-related depression, the idea of prophylactic depression treatment emerged[7]: when beginning interferon-alpha (and ribavirin) treatment, the patient would be started on an antidepressant with the goal of preventing, or attenuating, depressive symptoms. Initial case studies and case series noted success of this strategy. For example, antiviral treatment was restarted in a cohort of eight chronic hepatitis C patients who previously had discontinued due to emergent depressive episodes; all eight were able to fully complete the second course of treatment[8]. A precedent for this strategy was noting the success of antidepressant prophylaxis for interferon-alpha treatment of malignant melanoma[8,9].

Compared to prophylaxis, traditional practice can be termed "rescue" when depressive symptoms emerge in a patient undergoing antiviral treatment, depression treatment is quickly initiated so that those symptoms can be managed. The advantage to the prophylactic strategy is that depression and the threat of discontinuation can be avoided; the advantage to the rescue strategy is that patients are not unnecessarily treated, and so are not experiencing the additional treatment burden and side effects. Antidepressants may have quite adverse side effects in some patients, including retinal or gastroenterological bleeding[10,11]. Thus, clinicians are faced with a challenging clinical management strategy where risks and benefits must be considered.

Can prophylactic antidepressant treatment boost interferon-alpha treatment completion in patients with chronic hepatitis C virus (HCV)? No study has prospectively answered this question. This review has been conducted to discern an answer by reviewing antiviral therapy discontinuation data reported in trials evaluating the efficacy of antidepressant prophylaxis for managing depression. This review is presented in order to enhance the evidence available for clinical decision-making.

LITERATURE SEARCH

A Pubmed literature search was designed to discover trials that might have the data necessary to assess relative treatment completion between prophylaxis arms and control arms. A set of search terms was developed to capture studies relevant to hepatitis C. This included: "hepatitis", "HCV", "Hep C", "Hep-C" and "chronic hepatitis C". This was crossed with each of two other sets. The first was a set to capture depression-related studies: "depression", "depressed", "depressive", "psychiatric", "mental". The other was a set to capture prophylactic strategies: "prophylaxis", "prophylactic" or "prevention".

From this search, all study titles would be reviewed to detect promising abstracts. All promising abstracts would be read, and likely studies would be pulled and assessed for necessary information. References of those studies would be checked manually.

The necessary information for selection into this review was established as the following: patients with chronic hepatitis C who were candidates for interferon-alpha treatment (whether including ribavirin or not, as this treatment strategy emerged as the prophylactic strategy emerged); recognized treatment regimen (*i.e.*, interferon-alpha with ribavirin); no concurrent treatment such as for human immunodeficiency virus, since symptoms and treatment side effects would be significant confounders; at least two study arms where one included prophylactic treatment with an antidepressant, whether open-label or blinded, and the other is a control arm, whether placebo-controlled or not; sustained treatment of at least 8 wk in order to observe emergence of depressive symptoms from interferon-alpha and assess differential depression response between arms; and data on the numbers of patients in each arm that discontinued, or were lost to follow-up, for reasons other than medical side effects (thrombocytopenia, *etc.*) or non-response to antiviral therapy. Thus, the discontinuation group of focus would be those who medically could have completed treatment but discontinued for a reason other than a medical reason. To the degree that discontinuation reasons, such as psychiatric side effects, would be specifically reported, these would be tabulated and compared between the intervention-arm participants and the control-arm participants. The reporting of discontinuation for psychiatric reasons, specifically, was thus not an inclusion criterion.

For each eligible study, the number of patients discontinuing would be noted for each of the arms of the study. A descriptive analysis would be developed based on those results. The goal would be to describe the degree, if any, that antiviral treatment completion might be superior for the prophylactic strategy, compared side-by-side with the rescue strategy. Since the data sources for this study consisted of previously-published research studies, ethics approval for this narrative review was not sought from an institutional review board.

SEARCH RESULTS

For the "prophylaxis" search term set, "pretreatment" was soon discovered as a synonym, so this was added to that set. The "prophylaxis" set returned 1302661 abstracts; the "hepatitis C" set returned 184063 abstracts; and the "depression" set returned 869174 abstracts. The intersection of these three sets returned 419 abstracts. Titles of all were reviewed, leading to a set of 38 abstracts to review. This led to a set of 12 studies[8,12-23] in which the prophylactic strategy was evaluated, and discontinua-

Table 1 Study characteristics and discontinuation data: Antidepressant prophylaxis for interferon-alpha treatment of hepatitis C virus

Study	Leading exclusion criteria	Prophylactic intervention	Medication run-in period	Randomized: Yes/No	Blinded: Yes/No	Follow-up point, in wk[1]	Discontinued/Total in Arm:[2]	
							Prophylaxis	Rescue
Schaefer *et al*[12]	Psychiatric history, interferon treatment	Escitalopram	2 wk	Yes	Yes	24/48	2/90	3/91
Klein *et al*[13], Klein *et al* [14][4]	Active psychiatric disorder in the recent six months	Citalopram	3 wk	Yes	Yes	12	3/29	1/30
de Knegt *et al*[15]	Current Axis I disorder or current psychiatric prescription	Escitalopram	2 wk	Yes	Yes	24	2/34	2/37
Morasco *et al*[16]	Recent 3 mo psychiatric disorder	Citalopram	2 wk	Yes	Yes	24	N/A[3]	N/A[3]
Diez-Quevedo *et al*[17]	Active psychiatric disorder in the recent two months	Escitalopram	2 wk	Yes	Yes	24/48	4/51	6/48
Liu *et al*[18][5]	Alcohol use during treatment	Specific antidepressants not reported	Not reported	No	No	24/48	11/23	2/25
Neri *et al*[19]	Substantial psychiatric history, interferon treatment	Individual, family, and marriage counseling	N/A	Yes	No	24	14/106: 9/106 for lack of compliance, 5/ for psychiatric reasons	28/105: 11/105 for lack of compliance, 17/105 for psychiatric reasons
Gleason *et al*[20]	Depression not yet in remission	Escitalopram	4 wk	N/A	N/A	24/48	2/10	No control group
Morasco *et al*[21]	Active psychiatric disorder in recent six months	Citalopram	4 wk	Yes	Yes	24	3/13	3/15
Raison *et al*[22]	Psychiatric disorder or prescription within recent six months	Paroxetine	2 wk	Yes	Yes	24	0/18	6/18
Kraus *et al*[8]	Active substance abuse	Paroxetine or citalopram	3 wk	No		15	0/8	Not reported for control group of 9
Schaefer *et al*[23]	Current or recent psychiatric diagnosis or prescription	Citalopram	2 wk	N/A	N/A	24	0/14	0/11
						Totals:	47/396	45/380

[1]If 24/48, then treatment period accorded to interferon alpha regimen according to genotype, typically 24 wk for genotypes 2 and 3, and 48 wk for genotypes 1 and 4; [2]Denominator is total, including those lost to follow-up, who could have dropped out due to psychiatric reasons, and excludes those without this possibility, which would include the following: non-response to antiviral therapy, drop-out after randomization but before beginning antiviral therapy, or medical adverse events such as thrombocytopenia; [3]Three of 39 altogether discontinued antiviral therapy, but study report did not distinguish between active treatment and placebo for medical or other discontinuations; [4]2008 study was a trial design report, and 2012 was poster of initial results recently presented at a conference; [5]Retrospective observational chart review study; analysis limited to the two of four study arms that constitute a prophylaxis *vs* rescue comparison; 19 of the 23 in the prophylaxis arm were already taking antidepressants and the remaining 3 were started prophylactically before starting antiviral therapy.

tion data were reported.

These studies are listed, with relevant study characteristics, in Table 1. All but one were prospective trials; one was a retrospective chart review study that composed a cohort of patients who were taking an antidepressant before the initiation of interferon-alpha treatment, and composed a control group of patients who required some kind of psychiatric treatment during interferon-alpha treatment. For the sake of completeness, this chart review study was included. One of the 12 studies (Gleason *et al*[20], 2007), among the first chronologically, did not have a control group; this study simply investigated treatment completion when a prophylactic strategy was trialed. This was included for completeness. For one study, the manuscript reporting the preliminary study design was available, and results have just recently been presented as a poster at a scientific conference; it is assumed that a more complete analysis will be forthcoming. For the sake of completeness, results based on this conference poster were included.

Clinical Interventions

All studies were conducted in the era of prescribing ribavirin along with interferon-alpha. Nearly all were conducted in the era of pegylated interferon, with the exception of some of the earlier-initiated participants in the Morasco *et al*[21] (2007) and Raison *et al*[22] (2007) studies. Likewise, antidepressant dosages were normative, with typical strategies for increasing or augmenting dosage when clinically indicated, and typical medication switching strategies when clinically indicated. All studies used antidepressants from the selective serotonin re-uptake inhibitor class, including paroxetine (one study), paroxetine or citalopram (one study), citalopram (five studies), and escitalopram (four studies). This usage followed the pattern of Food and Drug Administration approval and clinical

adoption of these drugs, with paroxetine favored in earlier studies, citalopram favored in the studies conducted in the middle of this time span, and escitalopram favored in later studies. A range of strategies were used to assess depression level before and during treatment. These generally included: standardized clinical interview, clinical interview, a depression questionnaire, or combination. In some studies, patients could be started on antiviral therapy even if some level of depressive symptoms was present.

Clinical outcomes

The overwhelming majority of patients were able to complete interferon-alpha treatment. Sustained viral response results were in line with other well-managed intervention studies using interferon-alpha and ribavirin (*e.g.*, approximately 40% sustained viral response for those with genotype 1, approximately 75% for those with genotypes 2 or 3). Some patients failed to show a treatment response, and so interferon-alpha was discontinued due to lack of response. Some patients had treatment-related adverse events, such as thrombocytopenia, requiring discontinuation of therapy. To the degree that these data were available, the current study did not include these patients in the denominator at risk of discontinuing due to psychiatric difficulties, since they had discontinued due to medical reasons. Patients who were lost to follow-up or discontinued for other preference or discretionary reasons, or for unidentified reasons, were included in the numbers of patients who discontinued treatment for some reason other than antiviral non-response or medical side effect. This strategy was chosen because it can be challenging, especially from limited data included in published studies, to determine the leading reason for discontinuation or loss to follow-up, and the clinical question is whether prophylaxis boosts study completion.

Generally, providing antidepressant treatment resulted in amelioration of depressive symptoms. For the groups receiving antidepressant treatment prophylactically, average levels of depressive symptoms, or the portion of patients with an emergent depressive disorder, were lower in those receiving prophylactic treatment *vs* rescue treatment. Generally, problems with depression were worse for those at baseline with any depressive disorder history, or with higher initial depression severity.

Despite the clinical efficacy of antidepressant prophylaxis in controlling depressive symptomatology, there seemed to be no indication that the prophylactic strategy boosted treatment completion rates compared to the rescue strategy. Table 1 presents these data by study, including a summation of the total number of patients in the denominator, at risk for discontinuation, for both prophylactic and rescue arms, and the number for both arms that discontinued therapy. Of 396 patients in the prophylaxis arms altogether, who did not discontinue due to medical adverse events or clinical non-response, 47 (11.9%) discontinued interferon treatment before a recognized stopping point (*e.g.*, 24 or 48 wk); of 380 patients in the rescue arms, 45 (11.8%) discontinued interferon treatment. There was no overall statistical difference when tested by Chi-Squared test with Yates' correction (χ^2 = 0.00, P = 0.99).

One study (Raison 2007) seemed to yield a desired effect for prophylaxis: none of the 18 prophylaxis patients discontinued, while 6 of the 18 rescue patients discontinued. A review of this study in the context of other studies did not reveal any clear aspect of study design, measurement, or sampling that would indicate an explanation for this divergent result from the other, similar studies.

The Liu *et al*[18] study (2010) had greater discontinuation in the prophylaxis arm, but the psychosocial intervention used in this study, close monitoring and various counseling modalities, and psychopharmacotherapy only in certain cases where this psychosocial intervention was not successful, was very different from the other studies. Aside from this differential in discontinuation, the psychosocial intervention used in the Liu *et al*[18] study otherwise was successful in managing psychiatric symptoms, and doing so with less dependence on psychopharmacotherapy, compared to the usual care arm with rescue psychopharmacology. In this Liu study, with a psychosocial strategy for prophylaxis rather than psychaopharmacotherapy, the number of patients experiencing severe psychiatric symptoms was lower in the intervention group, with five meeting this criterion, *vs* 17 in the control group. Psychiatric symptomatology at less severe levels, likewise, was less frequent for the intervention arm compared to the control arm, with only six of the intervention patients eventually receiving antidepressant treatment compared to 19 in the control arm.

There were nine studies with data that permitted a Fisher's Exact Test to test whether the discontinuation rate differed between prophylaxis arm and rescue arm. Of these nine, only four had results that were statistically significant. Three modestly favored prophylaxis. These were: Diez-Quevedo *et al*[17] 2010 (7.8% discontinuation in prophylaxis arm, 12.5% rescue arm, Fisher's P = 0.02), Neri *et al*[19] 2010 (8.5% discontinuation in prophlylaxis arm, 10.5% discontinuation in rescue arm, Fisher's P = 0.02), and Raison *et al*[22] 2007 (0.0% prophylaxis arm, 33.3% rescue arm, Fisher's P = 0.02). The one study favoring rescue was Liu *et al*[18] 2010 (47.8% discontinuation in prophylaxis arm, 8.0% discontinuation in rescue arm, Fisher's P = 0.02). With five studies having no statistical difference in discontinuation, three favoring prophylaxis by varying portions, and one favoring rescue by a strong portion, there seems to be no consistent pattern favoring either strategy.

Since these studies were focused upon the presence and severity of depressive symptoms, but not on reasons for failure to complete a full course of therapy, reasons for not completing therapy were not systematically reported, and those reporting did not use consistent criteria. For those that did report, the stated reasons for discontinuation are listed in Table 2. Predominant reasons for not completing therapy included: Lost to follow-up, psychiatric side effects, and non-adherence. These reasons are likely quite overlapping, such as a person

Table 2 For studies reporting discontinuation data, number discontinuing interferon-alpha therapy, and reason for discontinuation, summed across studies

Reason for discontinuation	Prophylactic arm	Rescue arm
Lost to follow-up	21	19
Psychiatric side effects	8	18
Non-adherence	11	14
Did not complete therapy	3	3
Noncompliant or loss to follow-up	0	6
Other side effects	0	4

choosing to fail to continue in treatment due to psychiatric symptoms.

DISCUSSION

Emergence of depressive symptoms is a challenging side effect when treating chronic hepatitis C with interferon-alpha. Rates of depression may be as high as 30% or more. It has been established that monitoring patients for the emergence of depression, and rescuing those in whom depression emerges, is a successful strategy for limiting treatment discontinuation or poor adherence. Because of this high incidence of treatment-related depression, the idea of prescribing an antidepressant prophylactically to all patients at the initiation of antiviral therapy is attractive. This search revealed 12 studies that have evaluated the benefits of prophylactic treatment. From these studies, it is clear that prophylactic treatment serves to reduce the emergence of depression, and serves to manage the level of depressive symptomatology.

This review was undertaken to investigate the degree that the prophylactic strategy might boost treatment completion. There is no clear indication that the prophylactic strategy generally serves to boost treatment completion, compared to a monitor-and-rescue strategy. Where noted, nearly all patients in the rescue arms were successfully rescued from the emergence of depression. Review of study parameters does not suggest any treatment strategy or patient profile where prophylaxis yields a boost in treatment completion.

Advantages to prophylaxis are the superior management of depression during treatment in some portion of patients. This advantage needs to be weighed against the negatives of this strategy, which include the increased treatment burden on the patient, increased cost, and the risk of adverse events from the antidepressant. Two of the reviewed studies indicate some likely applications for prophylaxis. The study by Schaefer *et al*[12] (2005) demonstrated lower rates of treatment-related depression in the prophylactically treated arm, compared to the arm with no prophylaxis, in a cohort of patients with chronic hepatitis C who also had a history of a mental disorder (predominantly affective and dependence disorders) but with no active symptomatology and not currently receiving any psychiatric medication. The Kraus *et al*[13] (2005) study demonstrated successful interferon-alpha retreatment with antidepressant prophylaxis for a cohort of patients who had previously discontinued interferon-alpha treatment due to the emergence of depressive symptoms, while the control arm experienced, on average, even higher depressive symptom levels in the second attempt at interferon-alpha treatment (possibly due to the use, for all, of pegylated interferon-alpha in the second but not first treatment attempt). So, certain subgroups with recognized psychiatric difficulties may benefit from antidepressant prophylaxis.

While psychopharmacology is effective for managing depression in interferon-alpha treatment of hepatitis C, it is interesting to note the positive results of the Liu study, with a psychosocial intervention including individual counseling, family counseling, and couples counseling. The exact design of this intervention was not reported, such as how counseling needs were discovered, or data on the number of sessions delivered, or the specific clinical issues addressed, or whether any component included comprehensive chronic illness management training (disease education, treatment education, stress management, physician-patient communication skills, *etc.*), which has been shown to improve treatment adherence along with health-related quality of life.

Why didn't the prophylaxis approach have superior treatment completion, along with superior depression management, compared to rescue approach? It is possible that, in these trials, the rescue strategy worked as well as prophylaxis because clinical trials often have clinical management practices (answering patient questions, establishing clear lines of communication, systematic symptom monitoring, recruitment of motivated patients) that is stronger than usual care. If this is the case, then those delivering interferon-alpha treatment for chronic hepatitis C should be sure to parallel the symptom monitoring strategy of these trials. The monitoring of depression is a topic that has already been covered well in the literature concerning antiviral therapy, and has long been incorporated into treatment guidelines. The results of the Neri *et al*[19] (2010) study support this possibility: strong psychosocial monitoring led to better affective symptom control, with only a small portion of that advantage due to the use of antidepressants. At the same time, it is valuable to note that, in the Liu *et al*[18] (2005) study, interferon-alpha treatment conclusion or discontinuation led to a reduction in the emergent depressive symptom levels seen, leading the authors to conclude that "depression was specifically related to IFN therapy".

One indirect benefit of antidepressant treatment may be the management of treatment side effects other than psychiatric side effects. Raison *et al*[22] (2007) found stronger completion rates in the prophylaxis arm, and this was noted as being related to lower antiviral side effect difficulties. The study by Diez-Quevedo *et al*[17] (2010) also noted lower levels of antiviral side effects in those receiving antidepressants. Antidepressants are used in a range of clinical indications beyond depression, such as management of pain and management of fibromyalgia symp-

toms. In antiviral therapy, antidepressants may somehow reduce a range of symptoms. This could explain an unusual finding regarding depression in a larger hepatitis C study[24] that used a rescue strategy for emergent depression: while depression emerged for 90 patients in this study of nearly 400, discontinuation rates were lower for those patients (6%) than for those in whom no depression emerged (15%). The antidepressant intervention, or the related social support experienced in the course of clinical response, may have served to ameliorate the experience of treatment side effects. Data were not sufficient in the studies reviewed here to investigate more fully the possibility that antidepressant treatment in antiviral treatment may ameliorate antiviral-related side effects.

Another treatment characteristic suggesting that prophylaxis has limited clinical benefit was the necessity of monitoring and rescuing patients in the prophylaxis group, as well as the rescue group. In the de Knegt *et al*[15] study (2011), with 40 patients in the escitalopram group and 39 in the placebo group, four in the prophylaxis group needed rescue (increase or augmentation of dose, or new medication) while seven patients in the placebo group needed rescue depression treatment. In the Schaefer *et al*[23] (2012) study, three in the prophylaxis group needed rescue by another antidepressant, while 16 in the rescue arm required rescue. In the Morasco *et al*[21] (2010) study, approximately 30% in each arm had to have medication dosage adjusted, with some of those in the prophylaxis arm entering "rescue" treatment. This need to monitor and adjust pharmacotherapy is a limit to the treatment efficiency to be gained by prophylaxis; prophylaxis does not reduce the necessity of monitoring patients for the emergence of depression symptoms, and so does not greatly lighten the task of clinical care required to manage depression.

Because the influences of cytokines upon the central nervous system are quite varied, it is not quite clear how interferon-alpha causes depression in some patients. Pro-inflammatory cytokines can experimentally induce "sickness behavior" in non-human animals. It is hypothesized that this malaise might serve a valuable function: when the body needs to fight off infection, it is advantageous to have a healing period of increased sleep, lower activity level, and lower appetite; pro-inflammatory cytokines promote inflammatory responses, and also may simultaneously be registered in the brain, leading to the coincident sickness behavior[25]. Research in humans has revealed that interferon-alpha has an array of effects in the central nervous system, and elevated cytokine activity, especially tumor-necrosis factor-alpha and interleukin-6 can be noted in some portion of cases of major depression[26,27]. Further, serotonin-acting antidepressants have an effect upon tumor-necrosis factor-alpha and interleukin-6, as well as other inflammatory markers[28].

Providers should be clear about desired purpose when considering prophylactic antidepressant for hepatitis C patients about to begin antiviral therapy. Antidepressant prophylaxis does not seem to boost treatment completion, so other goals, such as managing depression, should be clarified when considering the strengths and weaknesses of this strategy. Discontinuation of interferon-alpha for chronic hepatitis C is a great treatment challenge, and anything that interferes with completion of treatment should be well investigated.

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MINIREVIEWS

Impact of PRRSV on activation and viability of antigen presenting cells

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important diseases of swine industry. The causal agent, PRRS-virus (PRRSV), is able to evade the host immune response and survive in the organism causing transient infections. Despite all scientific efforts, there are still some gaps in the knowledge of the pathogenesis of this disease. Antigen presenting cells (APCs), as initiators of the immune response, are located in the first line of defense against microorganisms, and are responsible for antigen recognition, processing and presentation. Dendritic cells (DCs) are the main type of APC involved in antigen presentation and they are susceptible to PRRSV infection. Thus, PRRSV replication in DCs may trigger off different mechanisms to impair the onset of a host effective immune response against the virus. On the one side, PRRSV may impair the basic functions of DCs by regulating the expression of major histocompatibility complex class Ⅱ and CD80/86. Other strategy followed by the virus is the induction of cell death of APCs by apoptosis, necrosis or both of them. The impairment and/or cell death of APCs could lead to a failure in the onset of an efficient immune response, as long as cells could not properly activate T cells. Future aspects to take into account are also discussed in this review.

Key words: Porcine reproductive and respiratory syndrome; Antigen presenting cells; Dendritic cells; Immune response; Major histocompatibility complex class Ⅱ; CD80/86; Cell death; Apoptosis

Core tip: Porcine reproductive and respiratory syndrome virus (PRRSV) is able to evade the host immune response and survive in the organism causing transient infections. PRRSV interacts with antigen presenting cells, specifically with dendritic cells, causing a regulation of major histocompatibility complex class Ⅱ and/or CD80/86 and cell death by apoptosis and/or necrosis.

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INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRS-virus (PRRSV)[1,2]. This virus belongs to the genus Arterivirus[3] and has a high genetic variability[4]. Two genotypes of PRRSV can be distinguished: PRRSV-1, which comprises strains from Europe; and PRRSV-2, which includes strains from America[3]. Indeed, PRRSV-1 is subdivided into three different subtypes, subtype 1, which includes strains from Western Europe, and subtypes 2 and 3, which comprise strains from

Eastern Europe[4]. Moreover, PRRSV-1 strains can also be classified in accordance with the capability to induce different patterns of tumour necrosis factor α (TNF-α), interleukin-10 (IL-10) after infection of peripheral blood mononuclear cells, porcine alveolar macrophages, peripheral blood SwC3$^+$ cells and bone marrow dendritic cells (BMDCs) into: IL-10$^+$-TNF-α$^+$, IL-10$^-$-TNF-α$^+$, IL-10$^+$-TNF-α$^-$ and IL-10$^-$-TNF-α$^-$-inducing strains[5].

Host immune response against the virus is weak and erratic and fails to control PRRSV. Different studies point out that type 1 interferons are insufficiently produced, which has been related to an inhibition of interferons by nonstructural proteins 1α, 1β, 2, 11, as well as, N protein[6-9]. Indeed, proinflammatory cytokines are also mildly produced[7,10,11] being associated nonstructural protein 2 to a decreased release of IL-1β and TNF-α[12,13]. Some *in vitro* evidences in the literature also point out that PRRSV can induce a suppression of NK cells[14]. Although PRRSV may induce the induction of cytotoxic T lymphocytes[15], these cells seem to suffer an impairment to exert their cytotoxic activity to PRRSV-infected macrophages[16]. Furthermore, the number of interferon-γ-secreting cells is not enough to control PRRSV[17-20] and neutralizing antibodies (NAs) are delayed and not produced in a vast extent[17]. Two to four weeks after infection, NA-response takes place, resulting in very low titers (1/32-1/64 or even lower)[17,18,20,21].

Antigen presenting cells (APCs) are located in the first line of defense against microorganisms attack. These cells recognize, process and present antigens to T cells in order to trigger an effective immune response[22-25]. While B cells can directly recognize antigens by means of its B cell-antigen receptor, T cells need the involvement of different molecules through two mandatory signals. The first signal consists on the binding between the T-cell antigen receptor (TCR) and the major histocompatibility complex class Ⅱ (MHC-Ⅱ) molecule. For the second signal, the CD28 molecule from T cells interacts with co-stimulatory molecules (CD80/86) from APCs. The correct linking of these molecules in the presence of antigens will suitably activate T cells[26,27].

Dendritic cells (DCs) are the main type of APC involved in antigen presentation. However, macrophages and B cells, although less efficiently, can also act as APCs[26,28-30]. Interestingly, it has been shown that different types of DCs and macrophages can suffer PRRSV replication *in vitro*[31-37]and *in vivo*[38-43]. However, in an *ex vivo* experiment, Loving *et al*[44] showed that lung-DCs were not permissive for PRRSV infection. A reasonable explanation for this result is that these lung-DCs could lack the receptors that PRRSV uses to go into the cell (*i.e.*, CD163, sialoadhesin, heparan sulphate)[45], while other types of DCs conserve these receptors. Furthermore, PRRSV replication directly impairs the basic functions of infected macrophages, including phagocytosis, antigen presentation and production of cytokines, and also induce cell death[46]. Therefore, changes in the number of APCs and/or a downregulation on the expression of MHC-Ⅱ and CD80/86 may lead to suppose an impairment in the onset of an effective immune response against PRRSV.

Other strategy followed by PRRSV to evade the host immune response might be the induction of cell death of APCs by apoptosis, necrosis or both of them[47-52]. Apoptosis is a regulated process modulated by both pro-apoptotic and anti-apoptotic cellular factors and it can be considered an active process[53], while necrosis is the passive death of cells[54]. In any case, APCs death could also cause a failure in the onset of an efficient immune response, owing to cells could not properly activate T cells.

WHAT DO WE KNOW ABOUT THE INTERACTION BETWEEN PRRSV AND APCS?

PRRSV and the expression of active (MHC-Ⅱ) and co-stimulatory (CD80/86) molecules

Due to the complexity in the isolation and culture of DCs from different porcine organs[55,56], the vast majority of conducted studies are *in vitro* studies.

After the infection of monocyte-derived dendritic cells (MoDCs) with either PRRSV-1 or PRRSV-2 strains, the expression of MHC-Ⅱ decreased[32,34,35] or remained unaltered[37]. The expression of MHC-Ⅱ in BMDCs infected with a PRRSV-2 strain did not show any change in its expression[33,36]. Nevertheless, according to Gimeno *et al*[5] in which 4 selected PRRSV-1 strains were used (one IL-10$^+$-TNF-α$^+$ strain, one IL-10$^-$-TNF-α$^+$ strain, one IL-10$^+$-TNF-α$^-$ strain and one IL-10$^-$-TNF-α$^-$ strain), infected-BMDCs exhibited either an increased expression of MHC-Ⅱ or no changes. Three out of four of these strains induced high expression of SLA-Ⅱ, while the IL-10$^-$-TNF-α$^+$-prototype strain did not evidence any change. Therefore, the use of different genotypes on different or the same subpopulation of APCs leads to different outcomes[5].

With regard to the expression of CD80/86 molecules, some authors pointed out a decrease in the expression of these molecules on MoDCs[34], while others mentioned an increased expression on these cells[35]. With regard to BMDCs, a decrease[5], no changes[5] and an increased expression of CD80/86[5,33,36] have been reported. Interestingly, in the article published by Peng *et al*[36], it was observed that both, bystander and PRRSV-infected cells, showed high expression of CD80/86 which may be associated with the release of soluble factors by infected cells or the engulfment of infected and/or apoptotic DCs. In fact, in the above mention study from Gimeno *et al*[5], the IL-10$^-$-TNF-α$^-$ prototype strain leads to the highest increase in the expression of CD80/86 in BMDCs while the double positive one, induced a decrease in CD80/86 compared to mock-infected group. It demonstrates that the behaviour of each strain can vary depending on the induced-cytokine profile.

The diminished expression of MHC-Ⅱ has also been linked to a lack of proliferation of leucocytes when co-

cultured with PRRSV-infected DCs, suggesting that PRRSV might modulate the immune stimulatory function of porcine DCs[35]. Moreover, in most of the above mentioned studies, only one of the two molecules (either MHC-Ⅱ or CD80/86) was increased and both of them are mandatory for the correct activation of T cells[26,27]. These findings highlight the complexity of the immune response against PRRSV, which may be triggering off different mechanisms to evade the host immune response not only in PRRSV-infected cells but also in bystander non-infected cells.

The *in vivo* expression of MHC-Ⅱ, as well as, CD80/86 on APCs has been poorly studied. In a study carried out by our research group, pigs which had been infected with a PRRSV-1 strain showed a decrease in the number of macrophages, as well as, the expression of MHC-Ⅱ in the tonsil, retropharyngeal and mediastinal lymph nodes compared to uninfected pigs[43]. In addition, a significant negative correlation was found between the expression of PRRSV antigen and the number of human leucocyte antigen-DR (HLA-DR) positive cells. Studying consecutive immunohistochemical sections, we observed that most of PRRSV antigen-positive cells were negative for HLA-DR antigen[43], pointing out a downregulation of MHC-Ⅱ in PRRSV infected cells.

PRRSV and cell death

Not only PRRSV could alter the expression of molecules involved in antigen presentation. Other way to abrupt antigen presentation is causing the death of APCs. Thus, concerning this point, several *in vitro* and *in vivo* studies have been conducted.

Concerning *in vitro* experiments, some authors observed apoptosis in bystander non-infected cells of American Type Culture Collection CRL11171 cell line[49] at the same time that other authors perceived co-localization of both, apoptotic and PRRSV antigens on macrophages and MARC-145 cells[57]. According to these authors, PRRSV is first able to provoke an anti-apoptotic state on cells suffering viral replication, inducing apoptosis later when the replication cycle has taken place. However, not only death has been observed during PRRSV infection, but also necrosis of MARC-145 cells[58,59].

Regarding MoDCs and BMDCs, cell death after PRRSV infection has been poorly studied. Both, apoptosis[32,34] and necrosis[32] phenomena have been noticed, although no co-localization of apoptotic or necrotic markers with PRRSV antigen were studied.

In vivo studies have evidenced apoptotic cells in testis[48], lungs and lymphoid organs of PRRSV-1 and PRRSV-2 infected pigs[49-52]. Although apoptosis has been associated with GP5 of PRRSV in infected cells[47,60], cell death has also been reported in non-infected bystander cells[49,50,52]. However, no co-localization of apoptotic markers and PRRSV expression has been analysed. This approach suggests that besides a direct induction of apoptosis by viral particles, an indirect pathway of apoptosis play a role in cell death during PRRSV infection. Several attempts have been carried out to relate indirect apoptosis of PRRS to the release of some apoptogenic cytokines, such as, TNF-α[61], IL-1 or IL-10[51]. Nonetheless, some of these associations could not be confirmed by *in vitro* studies with recombinant porcine cytokines[51]. Other studies have shown an enhanced expression of both Fas and FasLigand in PRRSV-2 infected splenic macrophages and in co-cultured splenic and peripheral blood lymphocytes[62], highlighting the necessity of exploring the role of different apoptotic mediators in PRRSV-induced cell death.

FUTURE ASPECTS AND ADVICES TO HEED IN THIS ISSUE

The expression of MHC-Ⅱ and CD80/86 has been analysed in different *in vitro* DC-models. However, these studies lack of the co-localization of PRRSV and the molecule involved in. Moreover, strains with different profile of cytokine release lead to different results. Therefore, co-localization studies, as well as, cytokine analyses should be performed in order to obtain clearer results on PRRSV modulation of the host immune response. Key cytokines might be interferon (IFN)-α and IFN-γ, because of their antiviral properties; TNF-α, due to anti-inflammatory, antiviral and apoptogenic functions; and IL-10, because of its immunomodulatory and apoptogenic properties. By doing so, it will be clarified if the virus itself, different cytokines, or both of them are able to cause a change in the expression of these molecules.

As above mentioned, TCR-MHC-Ⅱ and CD28-CD80/86 signals are mandatory to properly activate T cells. Thus, it is necessary to study both molecules in every conducted experiment to extrapolate and ensure the behaviour of these molecules.

A decreased expression of MHC-Ⅱ, CD80/86 or both of them could result in a failure or, at least, a non-effective immune response. In an *in vitro* study carried out in our group (data not published), it has been observed an enhanced expression of both molecules, MHC-Ⅱ and CD80/86, in MoDCs infected with a PRRSV-1 strain which had previously been tested for inducing a strong activation of the immune response. However, no proliferation of T cells was observed in this study and, on the contrary, a high rate of dead cells was detected. Therefore, a new strategy of the virus could be drawn, by which, although the virus induces the expression of MHC-Ⅱ and CD80/86 in MoDCs, they result ineffective since the virus later on induce their cell death. Thus, the use of cell-death markers should be also included in our routine experiments.

The same view should be extrapolated to death pathways studies. Moreover, future foresight experiments should broaden the spectrum of APC types and PRRSV strains in order to generate a clearer picture of this disease. The consideration of these aspects will improve the current knowledge on the pathogenesis and immune response against this virus, paving the way for its control.

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BRIEF ARTICLE

Genetic analysis of structural proteins in the adsorption apparatus of bacteriophage epsilon 15

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Abstract

AIM: To probe the organizational structure of the adsorption apparatus of bacteriophage epsilon 15 (E15) using genetic and biochemical methodology

METHODS: Hydroxylamine was used to create nonsense mutants of bacteriophage E15. The mutants were then screened for defects in their adsorption apparatus proteins, initially by measuring the concentrations of free tail spike proteins in lysates of cells that had been infected by the phage mutants under non-permissive growth conditions. Phage strains whose infected cell lysates contained above-average levels of free tail spike protein under non-permissive growth conditions were assumed to contain nonsense mutations in genes coding for adsorption apparatus proteins. These mutants were characterized by classical genetic mapping methods as well as automated sequencing of several of their genes. Finally, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography were used to examine the protein compositions of the radioactive particles produced when the various mutants were grown on a non-permissive host cell in the presence of ^{35}S-methionine and co-purified along with E15wt phage on CsCl block gradients.

RESULTS: Our results are consistent with gp4 forming the portal ring structure of E15. In addition, they show that proteins gp15 and gp17 likely comprise the central tube portion of the E15 adsorption apparatus, with gp17 being more distally positioned than gp15 and dependent upon both gp15 and gp16 for its attachment. Finally, our data indicates that tail spike proteins comprised of gp20 can assemble onto nascent virions that contain gp7, gp10, gp4 and packaged DNA, but which lack both gp15 and gp17, thereby forming particles that are of sufficient stability to survive CsCl buoyant density centrifugation.

CONCLUSION: The portal ring (gp4) of E15 is bound to tail spikes (gp20) and the tail tube (gp15 and gp17); gp17's attachment requires both gp15 and gp16.

Key words: Epsilon15; Virion structure; Salmonella phages

Core tip: Epsilon 15 (E15) is a temperate, serotype-converting bacteriophage that specifically infects group E1 Salmonellae bacteria. This paper presents genetic and biochemical evidence regarding the identities and positional relationships of the proteins that comprise the tail tube structure of E15. As such, it makes a small contribution towards what may someday be a fuller understanding, not only of how E15 stabilizes its packaged DNA, but also, how it triggers release of its DNA

when the phage encounters a susceptible Salmonella host cell.

Guichard JA, Middleton PC, McConnell MR. Genetic analysis of structural proteins in the adsorption apparatus of bacteriophage epsilon 15. *World J Virol* 2013; 2(4): 152-159 Available from: URL: http://www.wjgnet.com/2220-3249/full/v2/i4/152.htm DOI: http://dx.doi.org/10.5501/wjv.v2.i4.152

INTRODUCTION

Salmonella bacteria are enteric organisms that constitute a serious source of gastro-intestinal infection in humans and agriculturally important animals[1]. Bacteriophages provide an important mechanism of genetic variation and gene exchange among Salmonella bacteria (and thus, the potential for enhanced pathogenicity) through their ability to promote lateral transfer of host cell genes. Understanding the structural features of phage DNA packaging and adsorption/DNA ejection apparati is an important step in being able to fully assess how phage contribute to genetic variation within their Salmonella hosts.

Bacteriophage epsilon15 (E15) is a temperate, Group E1 Salmonella-specific phage that belongs to the Order "Caudovirales" and the Family "Podoviridae"[2]. At the genomic level[3], it closest relatives are the Salmonella-specific viruses, SPN1S (NCBI Accession number JN391180.1) and SPN9TCW (NCBI Accession number JQ691610.1) but it also shares 36 related genes in common with the *E. coli* O1H57-specific phage, ϕV10 (NCBI Accession number DQ126339.2). E15 was among the first Salmonella-specific phages to be discovered and was a popular experimental model for Japanese and US investigators in the 50's, 60's and 70's, both because of its ability to cause serotype conversion and because of its enzymatically active tail spikes, which display endorhamnosidase activity towards the host cell O-polysaccharide structure[4-9]. The publication of the E15 genome sequence by our laboratory in 2002 (NCBI Accession number AY150271.1) stimulated renewed interest in E15, this time as a model system for investigating virion structure by cryo-electron microscopy (cryo-EM), matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and other methods[3,10-14]. These studies, combined with earlier genetic and biochemical investigations[6], have revealed the following: (1) gp7 and gp10 together comprise the capsid of E15; (2) E15's enzymatically active tail spikes are homotrimers of gp20; and (3) other major proteins in E15 virions include gp4, gp15 and gp17. Circumstantial evidence, including size, relative abundance within virion particles and the position of its gene just downstream of those coding for the small and large terminase subunits in the late transcript are all consistent with gp4 being the portal protein of E15[3].

In addition to being a powerful tool for elucidating virion capsid structures, cryo-EM can also be used effectively to decipher the structure of a phage adsorption apparatus, especially if the adsorption apparatus can be detached intact from the virion capsid and prepared in purified form. Such was the case for the Group B Salmonella-specific phage, P22, and the resulting structure that was determined by cryo-EM analysis of these P22 adsorption apparati (termed "tail machines") is, in a word, spectacular[15,16]. To date, no one has reported having successfully purified the intact adsorption apparatus of phage E15.

In this paper, we present genetic and biochemical data that is consistent with gp4 forming the portal ring structure of E15; in addition, our data indicates that the centrally-positioned tail tube portion of the adsorption apparatus is likely comprised of gp15 and gp17, with gp17 being more distally positioned than gp15 and dependent upon both gp15and gp16 for its attachment. Finally, our data indicates that tail spike proteins comprised of gp20 can form stable associations with nascent virus particles that contain gp7, gp10, gp4 and packaged dsDNA, but which lack both gp15 and gp17. This implies that tail spikes bind directly to the portal ring during the assembly process that leads to the formation of mature virions.

MATERIALS AND METHODS

Phage and bacterial strains

Parental phages E15 and E15vir (a clear plaque mutant with a missense mutation in gp38, the major repressor protein) as well as bacterial host strains *Salmonella enterica subsp. enterica* serovar Anatum A1 *and Salmonella enterica subsp. enterica* serovar Anatum 37A2Su+ all came originally from the laboratory of Dr. Andrew Wright (Tufts University, Boston, MA). E15 (am2) is a nonsense mutant of E15 that is unable to produce tail spike proteins[6]. Propagation of bacteria and phage was in trypticase soy broth, unless otherwise indicated.

Isolation of phage nonsense mutants with adsorption apparatus defects

Nonsense mutants of E15vir were generated by hydroxylamine mutagenesis[17] and were detected initially by an anaerobic, double layer plating method that dramatically increases plaque size[18]. Hydroxylamine-treated phage were mixed with an amber suppressor strain (*Salmonella anatum* 37A2Su+) in the bottom LB soft agar layer, then overlaid with a second soft agar layer containing the non-suppressing parental strain *Salmonella anatum* A1. Turbid-looking plaques were cloned and re-screened to verify their inability to form plaques on *Salmonella anatum* A1.

Phage nonsense mutants isolated by the method described above were subsequently screened individually for potential defects in adsorption apparatus proteins other than the tail spike by measuring the level of free tail spike protein in lysates of non-permissively infected cells. The tail spike assay was based on a method developed earlier in an investigation involving phage P22 tailspikes[19]; It involved UV-irradiating 10000RPM (10K) supernatant frac-

tions obtained from lysates of *Salmonella anatum* A1 cells infected by E15vir nonsense mutants, then incubating the irradiated 10K supernatants with E15 "heads" obtained by infecting *Salmonella anatum* A1 with E15 (am2), an E15 nonsense mutant that is unable to produce tail spike protein. Following incubation, reaction mixes were plated at varying dilutions on the permissive host strain, *Salmonella anatum* 37A2Su+, in order to titer the number of E15 (am2) "heads" that were made infectious by the binding of tail spike proteins *in vitro*.

Genetic mapping and sequencing of Epsilon15 nonsense mutations: E15vir nonsense mutants isolated and screened as described above were characterized (along with the known tailspike nonsense mutant, am2) using classical *in vivo* complementation and two-factor recombination assay procedures that have been previously described[6]. These genetic mapping studies revealed the number of complementation groups (*i.e.,* genes) defined by the nonsense mutants and also allowed for an approximation of their locations relative to the E15 tail spike gene. Shortly after the mapping of the nonsense mutations using classical methods, the genomic sequence of E15 was completed by our lab. Gene 20 was then shown by sequencing analysis to contain the am2 nonsense mutation (*i.e.,* gp20 is the tailspike protein) and in addition, was observed to be the distal-most gene in the late mRNA transcript of E15[3].

Each E15vir mutant believed to be defective in an adsorption apparatus protein was subjected to DNA sequence analyses for genes 15, 16 and 17, in an effort to assign a gene identity for its nonsense mutation. The bracketing, Frwrd and Rvrse primer pairs used for initial PCR amplification of the three genes are shown below, with underlined bases representing modifications made in order to facilitate cloning of the PCR products into plasmids. Gene 15: *E15.Orf15*.Frwrd, AGGGATCCAAATGCCAGTTGTACCTACAG, *E15.Orf15*.Rvrse, ATACATAAGCTTTTATTCAACCCTCACG; Gene 16: *E15.Orf16*.Frwrd, TGGATCCATGGCTGATGTATTTTCACT, *E15.Orf16*.Rvrse, ACACATGCCTGCAGCATTATGGATTCCT; Gene 17*: E15.Orf17*.Frwrd, GAGGGATCCATAATGAAACAGGCATGTGT, *E15.Orf17*.Rvrse, GTTAAGGGTACCATCATTGTCCTA.

Because of their large sizes (ranging from 1928 to 2782 basepairs), the resulting PCR products were sequenced not only with the same Frwrd and Rvrse primers that had been used to produce them, but also with several additional primers known to bind internally within each PCR product. The internal sequencing primers were as follows: Gene 15: *E15.g15.W12689*: GGCGCTGCTCATGGCTGGAGTCATGAACAG, *E15.g15.W13264*: CGCGGCTATCGGTCTTTCTCAGTTACCTAC, *E15g15.W13879*: GGAGGCGGCTGCGCTGTCTGAACAGGTAC; Gene 16: *E15.g16.W15213*: CGGCAGGCATGGCCCTTCCTGCTGCTGTTG, *E15.g16:W15689*:TAGCGAACAGCCAGCGCATCCTGGATAAC; Gene 17: *E15.g17.W17092*: GCGGCAAAGTCTGCACAGTTCCAGATCCTG, *E15.g17.W17717*: GACCTGACGCTGCGCGAAACTTTTCCCTTG, *E15.g17.W18214*: GCGGCGTTCGGGCTGTTGATGTACAAAAAC.

Taq polymerase is somewhat error-prone[20], so in order to generate PCR products suitable for accurate DNA sequencing, PCR reaction mixes were prepared on a large scale (250 μL), then separated into five 50 μL aliquots prior to commencing the thermocycling reaction. Upon completion of PCR, the five aliquots were recombined into a single 250 μL sample and the DNA product was purified using a QIAGEN PCR purification column. Automated DNA sequencing reactions were performed by the Microchemical Core Facility at San Diego State University.

Preparation and analysis of ^{35}S-methionine labeled, virion-like particles produced by phage nonsense mutants under non-permissive conditions: Preparations of ^{35}S-methionine labeled, wild type E15vir phage particles and non-infectious, virion-like particles produced by the nonsense mutants were obtained by incubating mid-log phase *Salmonella anatum* A1 cells grown in low sulfate medium with phage (multiplicity of infection of 10) for ten minutes at 0 °C, then adding ^{35}S-methionine to a final concentration of 10 uCi/mL and shifting the incubation temperature to 37 ℃. At T = 90 min, cell cultures were lysed with chloroform, then centrifuged for 10 min at 10000 RPM in order to remove cellular debris. The resulting 10K supernatant fractions were loaded onto CsCl block gradients and centrifuged for 30 min at 38000 RPM on a Beckman L8-80M ultracentrifuge (an excess of cold E15wt phage was included in each sample as a carrier). Particles displaying virion-like densities (*i.e.,* the ability to pass readily through a 1.375 g/cm^3 CsCl layer and settle onto a 1.6 g/cm^3 CsCl layer along with non-radioactive E15wt carrier phage) were dialyzed, normalized for cpm and electrophoresed on 12% sodium dodecyl sulfate-protective antigen (SDS-PA) gels. The gels were subsequently dried on Whatman 3M paper and the paper was exposed to Kodak X-Omat X-ray film in order to detect radioactive proteins by autoradiography.

RESULTS

Isolation and mapping of E15 nonsense mutants with adsorption apparatus defects

We reasoned that cell lysates produced by infection of *Salmonella anatum* A1 with E15vir phage containing nonsense mutations in genes coding for adsorption apparatus proteins other than the tail spike should contain higher than normal levels of free tail spike protein. Cell lysates produced by infection with different E15 nonsense mutants were therefore screened for their ability to provide tail spike proteins to E15 (am2) "heads" *in vitro*, thereby rendering the heads infectious. Six E15vir nonsense mutants whose lysates had tail spike levels surpassing that

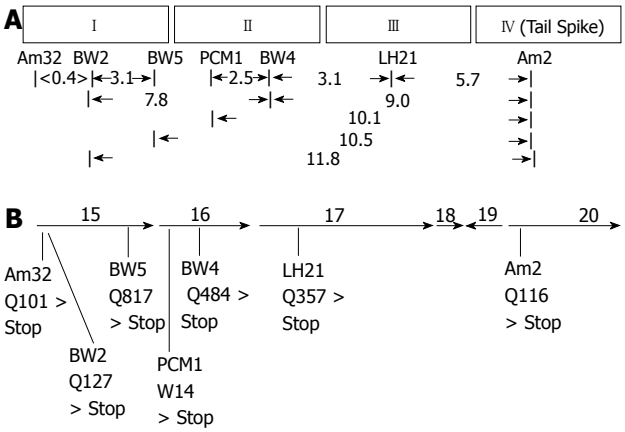

Figure 1 Genetic mapping and sequencing data showing positions of nonsense mutations that affect the protein composition of the epsilon 15 adsorption apparatus. A: Two-factor recombination values for nonsense mutations falling within *in vivo* complementation groups I through IV; B: Gene sequencing data. PCM1: Pericentriolar material 1; LH: Luteinizing hormone.

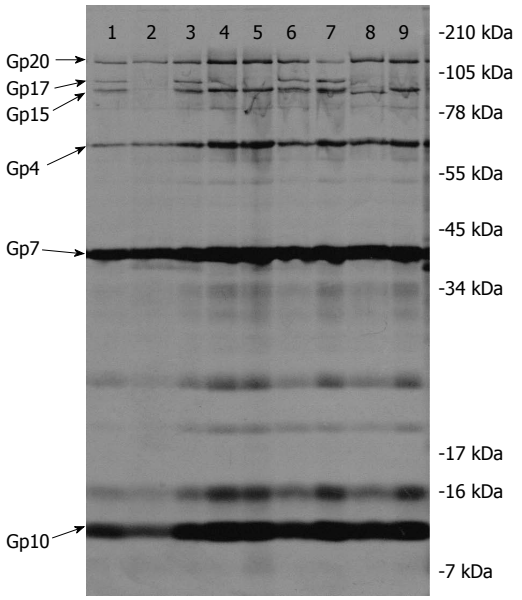

Figure 2 Autoradiogram showing compositions of non-infectious epsilon 15Vir particles. Lanes 1, 3 and 6, E15vir; Lane 2, gene 15 mutant am32 (BW2 is not shown but gives an identical pattern); Lanes 4 and 5, gene 16 mutants pericentriolar material 1 and BW4; Lane 7, partially suppressed am2 (gp20-) particles; Lane 8, gene 15 mutant BW5; Lane 9, gene 17 mutant luteinizing hormone21. molecular weight markers are depicted to the right.

of an E15vir lysate were identified, then further analyzed using classical genetic mapping methods. The six mutants were shown to define three complementation groups (*i.e.,* genes), which mapped in close proximity to each other as well as to the tail spike gene, defined by nonsense mutation am2 (Figure 1A). After confirming by DNA sequencing that the am2 mutation lay within gene 20 (the last gene in E15's "late" mRNA transcript), PCR primers were used to amplify and sequence three genes for each of the six mutants; namely 15, 16 and 17. Genes 15 and 17 were chosen for sequence analysis because the pI values, overall sizes, and tryptic digestion fragment sizes of their inferred polypeptide products closely matched those of E15 virion proteins shown by SDS-PA/autoradiography to be missing in virion-like particles formed by the various nonsense mutants under non-permissive conditions[3]. Gene 16 was included for sequence analysis as well because the genetic mapping data showed that the collection of six nonsense mutations with potential adsorption apparatus defects defined three different genes. Other neighboring genes (*i.e.,* 13, 14, 18 and 19) all coded for inferred proteins that were either very small or strongly hydrophobic, and were therefore not included in the sequencing analysis.

The DNA sequencing data (Figure 1B) revealed the presence of unique amber nonsense mutations in gene 15 for the three non-complementing phage mutants am32, BW2 and BW5. Non-complementing mutants pericentriolar material 1 (PCM1) and BW4 both contained unique amber nonsense mutations in gene 16, while mutant luteinizing hormone 21 (LH21), which the classical mapping data showed to be in a complementation group of its own, was found to contain a unique amber nonsense mutation in gene 17. The positions of the nonsense mutations determined by DNA sequencing correlated nicely with the linear map order that had been established for them previously by recombination analysis. In every case, the nonsense mutation had resulted from a hydroxylamine-induced C > T transition (either CAG > TAG, or TGG > TAG).

Yields and polypeptide compositions of E15 nonsense mutants with adsorption apparatus defects

MALDI-TOF mass spectrometry analyses of trypsin-digestion products obtained from purified E15 virion proteins[10] indicate that after the tail spike protein, gp20 (1070 amino acids, 115676 daltons), the next two largest proteins contained in E15 virions are gp17 (918 amino acids, 100841 daltons) and gp15 (842 amino acids, 91012 daltons). When ^{35}S-methionine-labeled particles produced by the various nonsense mutants under non-permissive conditions were co-purified with non-radioactive, "carrier" E15wt phage on CsCl block gradients, then analyzed by SDS-PAGE and autoradiography, it was observed that the two gene 16 mutants (PCM1 and BW4) and the gene 17 mutant (LH21) all produced good yields of radioactive particles relative to E15wt (118%, 154% and 100%, respectively, with a mean of 124 ± 28% SD) and that these particles all lacked gp17 (Figure 2, Lanes 4, 5 and 9). The three gene 15 mutants (am32, BW2 and BW5) all produced lower quantities of radioactive particles than E15wt (17%, 23% and 44%, respectively, with a mean of 28 ± 14% SD). The am32 and BW2 mutants, whose nonsense mutations mapped at codons 101 and 127, respectively, of gene 15 (845 codons), produced particles that lacked both gp15 and gp17 (Figure 2, Lane 2). Mutant BW5, whose nonsense mutation maps at codon 817 of gene 15, produced particles lacking gp17 but containing a novel protein with a slightly faster mobility than that of gp15; a protein most

likely comprised of amino acids 1 through 816 of gp15 (Figure 2, Lane 8). The quantity of the slightly truncated gp15 protein in BW5 particles is reduced, relative to the quantity of gp15 observed in E15vir and the various gp17-deficient mutants (see Lane 9, for example), thus indicating that its ability to assemble onto nascent virion particles has been diminished by the loss of 29 C-terminal amino acids, but not entirely eliminated. The 10K supernatant fractions obtained from cells infected by the three gene 15 mutants (am32, BW2 and BW5) were also analyzed by SDS-PAGE and autoradiography. All three supernatants contained a protein that co-migrated with the gp17 protein of E15wt (data not shown).

The two gene 16 nonsense mutants analyzed in this study (PCM1 and BW4) both produced good yields (118% and 154%, respectively, relative to wt E15) of non-infectious, virion-like particles that are missing gp17 (Figure 2, Lanes 4 and 5). As was the case for the three gene 15 mutants, a protein with gp17-like mobility was present in the 10K supernatant fractions of cells infected by PCM1 and BW4 (data not shown).

Every nonsense mutant that was studied produced radioactive particles that contained DNA, as judged by their ability to co-sediment with E15wt virions through CsCl at 1.375 g/mL and layer onto the 1.6 g/mL solution. In addition, all of the mutants, whether gp17-deficient or both gp15- and gp17-deficient, displayed normal quantities of the two known capsid proteins, gp7 and gp10, as well as gp4. Yields of the radioactive particles that lacked both gp15 and gp17 were significantly lower than those of particles that lacked gp17 only, suggesting that maximum stability of packaged DNA is achieved when both gp4 and gp15 are present. All of the mutant phage particles contained sufficient gp20 tail spike protein for easy detection by autoradiography (see lanes 2, 4, 5, 8, 9 of Figure 2).

DISCUSSION

The complete absence of both gp15 and gp17 in high-density particles produced by mutants am32 and BW2, whose nonsense mutations both map near the beginning of gene 15, combined with the gp17-only deficiency observed in high density particles produced by the gene 17 nonsense mutant (LH21), argues for a model in which gp15 and gp17 occupy penultimate and terminal positions, respectively, within a peripheral E15 virion structure that we hypothesize is the tail tube. The missing 29 amino acids at the C-terminal end of the gp15-like protein that is produced by BW5 phage under non-permissive conditions must be critical for gp17 binding since no gp17 protein was detected in these particles.

We currently do not know why gp16 is required for gp17's assembly onto nascent virions. The gp16 protein is inferred to have 634 amino acids and our two gene 16 nonsense mutations, PCM1 and BW4, are positioned at codons 14 and 484, respectively. The predicted mass for gp16 is 67364 daltons and its inferred overall methionine content (2.4%) falls within the range of methionine contents inferred for the other known virion proteins (from as low as 1.3% for gp20 to as high as 5.2% for gp4). In other words, if gp16 is present in E15 virions in appreciable quantities, then it should contain sufficient ^{35}S-methionine to show up in our autoradiogram. Faint protein bands were observed above the 78 kDa marker and above and below the 55 kDa marker on the gel (Figure 2), but none of these three proteins appeared to be diminished in quantity in the gene 16 mutants, relative to the other mutants or to E15vir. It is conceivable that gp16 is a virion protein that was not detected in our experiment because it co-migrated with gp4 protein (the inferred mass for gp4 is 61657 daltons). If that is true, though, one can argue that the quantity of gp16 in virions must be quite small, since the intensities of the gp4 bands in the two gene 16 mutants do not appear to be diminished, relative to those of E15vir and the other nonsense mutants that were analyzed. It should be noted that both our lab and at least one other have detected gp16 tryptic fragments in purified E15 virions using MALDI-TOF analysis[10]; the other lab has more recently hypothesized that gp16 is a tail tube protein[21]. While the data in this paper does not support that hypothesis, we remain open to the possibility and are continuing to explore the role played by gp16 in E15 virion assembly. It has also been hypothesized that gp17 functions as a pilot (or ejection) protein for E15[21]; this seems highly unlikely since ejection proteins, as the name implies, exit the capsid along with the DNA during the infection process[22,23]. Our results clearly show that E15 particles lacking gp17 retain stably packaged DNA within their capsids, as evidenced by their ability to co-purify in high yields with E15wt carrier phage on CsCl block gradients; furthermore, the same holds true, albeit to a lesser degree, for particles that are lacking both gp15 and gp17.

Frankly, we were surprised that tail spikes were present in all of the particles produced by our nonsense mutants. The initial screening procedure used to identify nonsense mutants for this study was based on the assumption that mutations resulting in adsorption apparatus defects would hinder tail spike assembly onto the virion, thereby resulting in higher than normal levels of free tail spike protein in the infected cell lysates, as well as the production of phage particles lacking tail spike proteins. Our current explanation is that gp4 forms the portal ring structure and perhaps, with help from immediately adjacent capsid proteins, provides a significant part of the binding surface(s) to which gp20 tail spikes normally attach during virion assembly. Interestingly, in their first cryo-EM paper dealing with E15, Jiang *et al*[10] reported that two of E15's six tail spikes occupy positions around the tail tube that place them in very close contact with the capsid. If these two tailspikes are more firmly bound in gp17- and gp15-/gp17-deficient particles than the other four, then that might explain both the presence of gp20 in the mutant particles as well as the enhanced levels of tail spike protein in their infected cell lysates.

Figure 3 sums up our current model for the structure of the E15 adsorption apparatus: (1) gp4 forms the

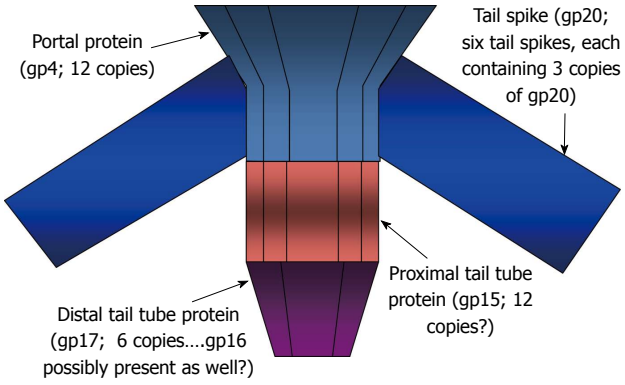

Figure 3 Schematic model for protein positions and interactions within the adsorption apparatus of bacteriophage Epsilon 15. The estimates of 12 and 6 copies for gp15 and gp17, respectively, are based upon stoichiometric measurements made relative to the numbers of capsid and tail spike proteins present in epsilon 15[13]; tail spike attachment to portal protein may be further stabilized by interactions with gp15 and/or capsid proteins.

portal ring structure and perhaps, with help from neighboring capsid proteins, provides a binding surface that is sufficient for attachment of tail spikes (gp20); (2) gp15 and gp17 form the central tail tube, with gp17 occupying the more distal position and interacting with gp15 by 4° interactions that cannot occur if the C-terminal 29 amino acids of gp15 are missing. The association of gp17 with gp15 is also gp16-dependent but we do not know yet whether or not gp16 forms part of the tail tube. We are currently continuing our study of E15 adsorption apparatus structure and function by conducting phenotypic suppression experiments with an E15 mutant in our collection that under non-permissive conditions, adsorbs to cells and degrades O-polysaccharide normally, but fails to eject its DNA[6].

The best understood Salmonella-specific phage in the Podoviridae family is P22 and recent X-ray crystallography and cryo-EM studies have revealed features of the proteins that comprise its capsid, portal, tail tube, needle and tail spikes in exquisite detail[15,16,24,25]. The dodecameric, ring-shaped portal structure of P22 is comprised of gp1; below the portal ring is the tail tube, comprised of twelve copies of gp4 (bound directly to the portal) and six copies of gp10, which are bound to gp4. Attached to the distal portion of gp10 is P22's "needle" structure, which is comprised of three copies of gp26. The six laterally-positioned, homo-trimeric tail spikes of P22 are comprised of gp9 and are thought to be associated with a binding surface generated cooperatively by proteins gp4 and gp10 at their point of junction on the sides of the tail tube[15].

Gene homology studies indicate that of the three Podoviridae phages known to infect Group E Salmonellae, namely E15, Epsilon34 (E34) and g341, two (E34 and g341) likely have adsorption apparatus protein compositions and organizations that are similar to that of P22[26,27]. Phage E15, on the other hand, has clearly taken a different path; Its tail spike protein is gp20, which at 1070 amino acids (aa) is about 63% larger, on average, than those of E34 (606 aa), g341 (705 aa) and P22 (667 aa) and is homologous with them only in a short stretch of amino acids at the N-terminal end that are thought to be critical for assembly onto the virion. Although they appear to occupy similar positions in the tail tube, there is no apparent structural homology between the proximal tail tube proteins of E15 and P22 (gp15 and gp4, respectively) or between their distal tail tube proteins (gp17 and gp10, respectively). There are stoichiometric similarities, though, in that densitometry measurements of Coomassie Blue-stained proteins of wild type E15 virions, followed by normalization for size differences, indicate that tail spikes (gp20), proximal tail tube proteins (gp15) and distal tail tube proteins (gp17) are present in E15 virions at approximately a 3/2/1 ratio, which matches the well-established 18/12/6 ratios of tail spike (gp9), proximal tail tube (gp4) and distal tail tube (gp10) proteins known to be present in P22 virions. No homolog of the P22 "needle" protein (gp26) is present among inferred bacteriophage E15 proteins, but that is not surprising since the tail tubes of negatively-stained E15 virions do not display the "needle-like" protuberance that is seen in electron micrographs of P22[6]. The "needle" is thought to play a role in the movement of the P22's genome across the bacterial cell envelope during an infection[28]. How E15 compensates for its lack of a "needle" protein remains to be determined.

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COMMENTS

Background

In April, 2010 (http://www.hhs.gov/asl/testify/2010/04/t20100428b.html), the Director of the Center for Disease Control reported to the House Subcommittee on Human Health that approximately 1.4 million Americans are infected annually with foodborne strains of Salmonellae bacteria. He further stated that the incidence of antibiotic resistance among isolates of Salmonella strains obtained in hospitals, stock animals and the food supply were all on the rise. Generalized transduction by bacteriophages is a major method for the horizontal transfer of genes between Salmonella organisms and thus, likely plays a role in their evolving pathogenicity.

Research frontiers

The ability of a bacteriophage to infect a bacterium is governed by the nature of its adsorption apparatus. The adsorption apparatus is a collection of proteins that cooperate together to maintain the stability of the phage's packaged genome until the moment when a susceptible host cell is encountered. At that point, the same sets of proteins interact with each other in an entirely different manner to trigger ejection of the phage genome and facilitation of its transport into the host cell cytoplasm.

Innovations and breakthroughs

Recent cryo-electron microscopy studies on virions of the Group E1 Salmo-

nella-specific bacteriophage, epsilon 15 (E15) have yielded highly detailed information on the composition and structure of the phage's capsid. Those same investigators have also produced the first close-up view of the adsorption apparatus of E15. This paper presents data regarding the identities of the proteins that comprise E15's adsorption apparatus; in addition, the data presented herein provides some insight into the ways these proteins interact with each other in order to form the adsorption apparatus.

Applications

Compared with other salmonellae-specific members of the podoviridae family, bacteriophage E15 appears to be unique when it comes to the collection of proteins that comprise its adsorption apparatus. Perhaps, in addition to the uniqueness of their physical characteristics, the manner in which these proteins interact with each other to control the stability of packaged DNA as well as its release in response to the proper environmental cue will also prove to be novel, and thus, worthy of further study.

Terminology

Adsorption apparatus pertains to those proteins that are stably associated with the mature virion, either through direct binding interactions with the portal ring or else, by virtue of their association with other proteins that are bound to the portal ring.

Peer review

The authors used genetic and biochemical methods to examine compositional and organizational aspects of the adsorption apparatus of bacteriophage E15. Although preliminary, the results are sufficient for establishing a simple model that should be possible to refine with further experimentation.

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BRIEF ARTICLE

Evolution of an avian H5N1 influenza A virus escape mutant

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Abstract

AIM: To investigate the genetic constitution of an escape mutant H5N1 strain and to screen the presence of possible amino acid signatures that could differentiate it from other Egyptian H5N1 strains.

METHODS: Phylogenetic, evolutionary patterns and amino acid signatures of the genes of an escape mutant H5N1 influenza A virus isolated in Egypt on 2009 were analyzed using direct sequencing and multi-sequence alignments.

RESULTS: All the genes of the escape mutant H5N1 strain showed a genetic pattern potentially related to Eurasian lineages. Evolution of phylogenetic trees of different viral genes revealed the absence of reassortment in the escape mutant strain while confirming close relatedness to other H5N1 Egyptian strains from human and avian species. A variety of amino acid substitutions were recorded in different proteins compared to the available Egyptian H5N1 strains. The strain displayed amino acid substitutions in different viral alleles similar to other Egyptian H5N1 strains without showing amino acid signatures that could differentiate the escape mutant from other Egyptian H5N1.

CONCLUSION: The genetic characteristics of avian H5N1 in Egypt revealed evidence of a high possibility of inter-species transmission. No amino acid signatures were found to differentiate the escape mutant H5N1 strain from other Egyptian H5N1 strains.

Key words: Chicken; Genotyping; H5N1; Influenza; Virus evolution

Core tip: The evolution of phylogenetic trees of different viral genes revealed the absence of reassortment in the examined escape mutant H5N1 strain; however, a variety of amino acid substitutions were recorded. The displayed amino acids substitutions in different viral alleles denoted considerable possibility of inter-species transmission, virulence to mammalian species and cytokine resistance.

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INTRODUCTION

The influenza A viruses belong to the Family Orthomyxoviridae. The hemagglutinin (*HA*) and neuraminidase (*NA*) genes encode viral envelope proteins and there are 17 *HA* and 10 *NA* subtypes[1]. Other influenza genes include *PB2*, *PB1*, *PA*, *NS*, *M* and *NP* that encode for viral internal proteins, are required for viral replication and assembly[2] and play an important role in viral infectivity[3]. Reassortments between different influenza A subtypes H9N2 and H5N1 or H7N3 have been detected[4,5]. Interspecies transmission can lead to catastrophic consequences. Egyptian H5N1 viruses are classified as clade

Table 1 Oligonucleotides used for amplification of the *H5N1* genes

Locus	Name	Primer sequence	Length	Amplicon size (bp)	Location	Ref.
N1	N1-F	ATGAATCCAAATCAGAAG	18	1350	21-38	[38]
	N1-R	TGTCAATGGTGAATGGCAAC	20		1346-1365	
PB2	B2-F	GAGGCGATCTGAATTTCG	18	986	1256-1273	[39]
	B2-R	TATGCTAGAGTCCCGTTTCC	20		2222-2241	
PB1	B1F	AGCGAGGAGTATCTGTGAGA	20	601	774-793	[40]
	B1R	TTCCCTCATGATTCGGTGCA	20		1356-1375	
PA	PA-F	ATGAAGAGAGCAGGGCAAGA	20	868	491-510	This study
	PA-R	CAATGGGATACTTCCGCTGT	20		1339-1358	
NP	NP-F	TGCTTGCCTGCTTGTGTGTA	20	665	823-842	[39]
	NP-R	TACTCCTCTGCATTGTCTCCGA	22		1466-1487	
M	M-F	CCCTCAAAGCCGAAATCGCGCA	22	875	56-77	[40]
	M-R	TGCTGTTCCTGCCGATACTCTTCCC	25		906-930	
NS	NS-F	CACTGTGTCAAGCTTTCAGG	20	798	23-42	[39]
	NS-R	TCTCTTGCTCCACTTCAAGC	20		786-805	

2.2.1, which is further subdivided into two groups: A (A1-A5) and B (B1-B2)[6]. The economic consequences, in addition to the zoonotic implications, of highly pathogenic avian influenza virus H5N1 continue to constitute an important problem. According to the recent report of the World Health Organization in June 2013, 628 H5N1 infected cases with 374 fatal consequences were recorded. Egypt is among the countries that contain a very high number of the infected human cases (172) with a total of 62 fatal cases[7]. Endemic situations of H5N1 in Egypt is still an unsolved problem[8]. In Egypt, vaccination of poultry with inactivated vaccine preparations is currently adopted to combat H5N1; however, vaccination of household poultry was suspended in mid 2009 due to the limited impact on H5N1 incidence[8]. In turn, so-called "escape mutants" resulting from antigenic drift of the viruses are selected[9,10]. Escape mutants are known to be less liable to neutralizing antibodies induced by vaccines. Influenza viruses showed a considerable capacity to cross species barriers and to infect and be transmitted among susceptible mammals, including humans. Point mutations and allelic combinations possess a crucial effect on the virulence of HPAI H5N1 isolates and are thought to be polygenic[11,12]. Genetic reassortments among avian influenza viruses are commonly detected in wild bird and poultry isolates[13,14]. The possibility that an avian influenza virus, H5N1, can evolve to human-to-human or mammal-to-mammal transmission through the acquisition of genetic material from the other influenza viruses subtypes already circulating in human or mammals is not weak. The currently studied strain is an escape mutant strain that belongs to 2.2.1, B2 sublineage[10]. The current study aimed to investigate the genetic constitution of the escape mutant strain and compare it with other influenza strains. It also aimed to screen the presence of possible amino acid signatures that could differentiate the escape mutant from other Egyptian H5N1.

MATERIALS AND METHODS

Viral RNA extraction and RT PCR

Viral RNA was extracted from the infective allantoic fluid of A/chicken/Egypt/F10/2009 using a spin column purification kit (Koma Biotech. Inc., South Korea). Amplification of viral genes was performed with gene-specific primers for *PB2*, *PB1*, *PA*, *NP*, *NA*, *M* and *NS* (Table 1) using a Koma one step RT PCR kit (Koma Biotech. Inc., South Korea). Following electrophoresis in a 1.5% agarose gel, bands of expected sizes were excised and purified using a QIAquick gel extraction kit (Qiagen, Germany). Purified amplicons were sequenced in both forward and reverse directions (Macrogen, South Korea). Sequences from different genes were routinely assembled and processed. Sequence data of the current study were submitted to the GenBank after removal of trimming primer-linker (Accession No. KC815941-KC815947).

Genetic and phylogenetic analysis

Sequence analysis of the viral genes was conducted using Mega 4.1 as previously described[15]. Sequence alignments of each of the seven genomic segments were conducted using the partial coding regions. Phylogenetic analyses of the A/chicken/Egypt/F10/2009 strain in the current study were conducted with other influenza A viruses to screen the possible reassortant allele. All gene sequence data were collected from the National Center for Biotechnology Information flu database. The neighbor-joining method with Kimura two-parameter distances was used for building the phylogenetic trees using the Mega 4.1[15]. The consistency of the internal branches was evaluated by the p-distance substitution model and 1000 bootstrap replications. The influenza A virus genotype tool at http://www.flugenome.org/genotyping.php[16] was used to determine individual genome segment lineages. A number of human, non-human mammalian and avian viruses were included in the evolutionary trees of *PB2, PB1, PA, NP, NA, M* and *NS* genes with selected sequences from different influenza serotypes in the GenBank to investigate relatedness and possible genetic reassortment.

Deduced amino acid sequence analysis

The multisequence alignment tool available in the flu database was used to compare the deduced amino acid

Table 2 Comparison of amino acid signatures in selected genes of avian and human strains to Egyptian H5N1 strains

Gene	Residue	Avian[1]	Human	Egyptian H5N1	A/CK/Egypt/F10/2009
PB2	475	$L^{214}M^{1}$	$M^{839}L^{3}$	$L^{52}M^{2}$	L
	588	$A^{203}/T^{6}/V^{6}$	$I^{835}/V^{3}/A^{2}$	A^{53}/T^{1}	A
	613	V^{212}/A^{3}	$T^{816}/I^{16}/A^{8}/V^{1}$	V^{54}	V
	627	E^{196}/K^{19}	$K^{838}/R^{2}/E^{1}$	K^{48}/E^{6}	K
	674	$A^{204}/S^{6}/T^{2}/G^{2}/E^{1}$	$T^{836}/A^{2}/I^{2}/P^{1}$	A^{54}	A
PB1	327	R^{147}/K^{3}	K^{766}/R^{66}	R^{58}	R
	336	V^{142}/I^{8}	I^{773}/V^{59}	V^{58}	V
PA	225	$S^{213}C^{1}$	$C^{829}S^{10}$	S	S
	268	L^{214}	$I^{827}K^{11}$	L	L
	356	$K^{212}X^{1}R^{1}$	$R^{827}K^{11}$	K	K
	382	$E^{208}D^{5}V^{1}$	$D^{824}E^{11}V^{2}N^{1}$	E	E
	404	A^{214}	$S^{828}A^{9}P^{1}$	A	A
	409	$S^{189}N^{24}I^{1}$	$N^{830}S^{7}I^{1}$	$S^{77}N^{1}$	S
NP	283	L^{372}/P^{1}	L^{7}/P^{643}	L^{61}	L
	293	R^{371}/K^{2}	R^{28}/K^{622}	R^{60}/K^{1}	R
	305	R^{369}/K^{4}	K^{636}/R^{14}	R^{61}	R
	313	$F^{371}/I^{1}/L^{1}$	Y^{642}/F^{8}	F^{61}	F
	357	$Q^{368}/K^{4}/T^{1}$	$K^{44}/R^{8}/Q^{1}$	Q^{61}	Q
	372	$E^{357}/D^{15}/K^{1}$	D^{630}/E^{23}	E^{61}	E
	422	R^{373}	K^{630}/R^{23}	R^{61}	R
	442	T^{372}/A^{1}	$A^{629}/T^{23}/R^{1}$	T^{61}	T
	455	D^{373}	$E^{630}/D^{22}/T^{1}$	D^{60}/E^{1}	D
M1	115	$V^{856}/I^{2}/L^{1}/G^{1}$	I^{981}/V^{9}	V^{88}	V
	121	$T^{840}/A^{19}/P^{1}$	A^{988}/T^{2}	T^{88}	T
	137	$T^{859}/A^{1}/P^{1}$	A^{974}/T^{12}	T^{88}	T
M2	11	$T^{434}/I^{11}/S^{2}$	I^{911}/T^{44}	T^{90}	T
	20	S^{471}/N^{13}	N^{926}/S^{29}	S^{90}	S
	57	$Y^{481}/C^{1}/H^{1}$	$H^{913}/Y^{33}/R^{2}/Q^{1}$	Y^{90}	Y
NS2	70	$S^{453}/G^{21}/D^{1}$	G^{903}/S^{2}	S^{61}	S

[1]Avian and human amino acid signatures in different viral genes of influenza A viruses as previously determined[20]. Numerical superscripts refer to the number of strains that possess those residues.

Table 3 Amino acid site residues associated with virulence in mammals in comparison with Egyptian isolates

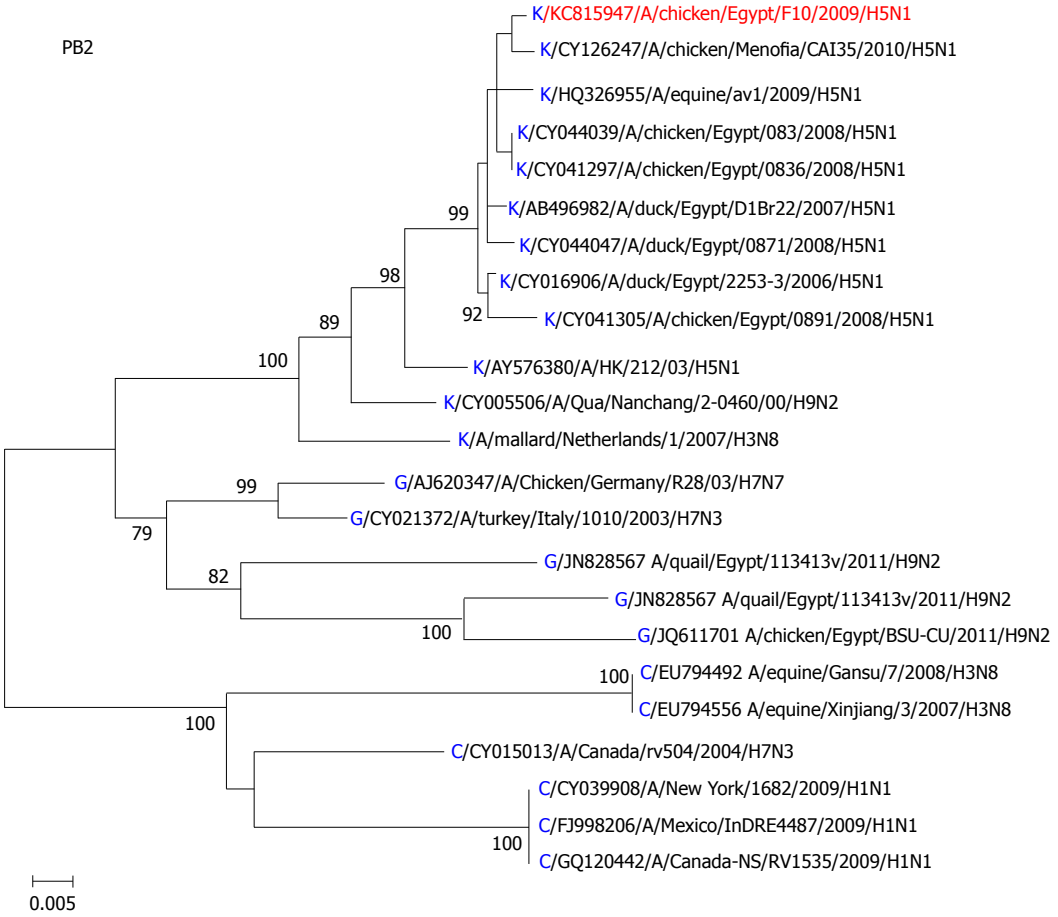
Gene	Site	Residue[1]		Egyptian	A/CK/	Ref.
		Virulent	Avirulent	H5N1 isolates	Eg/F10/09	
PB2	627	K	E	K	K	[11,27]
	701	N	D	D	D	[28]
PB1	317	I	M/V	M/V	M	[11,27]
PA	127	I	V	V	N.I.[2]	[25]
	336	M	L	L	L	[25]
M2	64	S/A/F	P	S	L	[17]
	69	P	L	P	P	[17]
NS1	42	S	A/P	S	S	[29]
	92	E	D	D	D	[27]
	97/92	E	D	E	E	[23]
	127	N	T/D/R/V/A	T/I	I	[30]
	189	N	D/G	D	D	[25]
	195	T/Y	S	S	S	[31]
NS2	31	I	M	M	M	[25]
	56	Y	H/L	H	H	[25]

[1]Virulent and non virulent amino acid residues refer to the ability of the virus to replicate in mammals as determined by Lycett *et al*[17]. [2]N.I.: Not included since it is not flanked by the primers used in the current study.

sequences of the seven genes of the A/chicken/Egypt/F10/2009 strain with other H5N1 strains from the Egyptian H5N1 isolates available in the flu database in order to screen amino acid signature and mutation trend change. Amino acid residues that have associated with mammalian virulence were also screened.

RESULTS

A/chicken/Egypt/F10/2009 in the current study is related to B2 sublineage. Eight amino acid substitutions were found in the F10 strain at the amino acid positions P74S, D 97N, H110R, S123P, R140G, F144Y, N165H and A184E. The different alleles of the F10 isolate were located within subtrees of the majority of the Egyptian strains (Figure 1). The influenza genotyping web tool revealed that the alleles of the F10, *PB2*, *PB1*, *PA*, *NP*, *NA*, *M* and *NS* alleles, are related to K, G, D, F, 1J, F and 1E genotypes respectively. Analysis of the *NA* gene revealed the presence of the 20-amino acid deletion (data not shown) and the presence of amino acid arginine (R) at position 110. The 228 (N to S) substitution is also present in the F10. The six internal genes (*PB2*, *PB1*, *PA*, *NP*, *M* and *NS*) of A/chicken/Egypt/F10/2009 showed avian like amino acid signatures (Table 2). The polymorphic amino acid residues in different protein sequences of the Egyptian human and avian strains in comparison to the current escape mutant strain were screened and the residues were classified as virulent or nonvirulent (Table 3). Five virulent residues were detected in the avian H5N1 strains in *PB2* (K627), *M2* (S64, P69) and *NS1* (S42, E92/97); however, F10 showed only 4 virulent residues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89
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G/CY021372/A/turkey/Italy/1010/2003/H7N3
79
G/JN828567 A/quail/Egypt/113413v/2011/H9N2
82
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100
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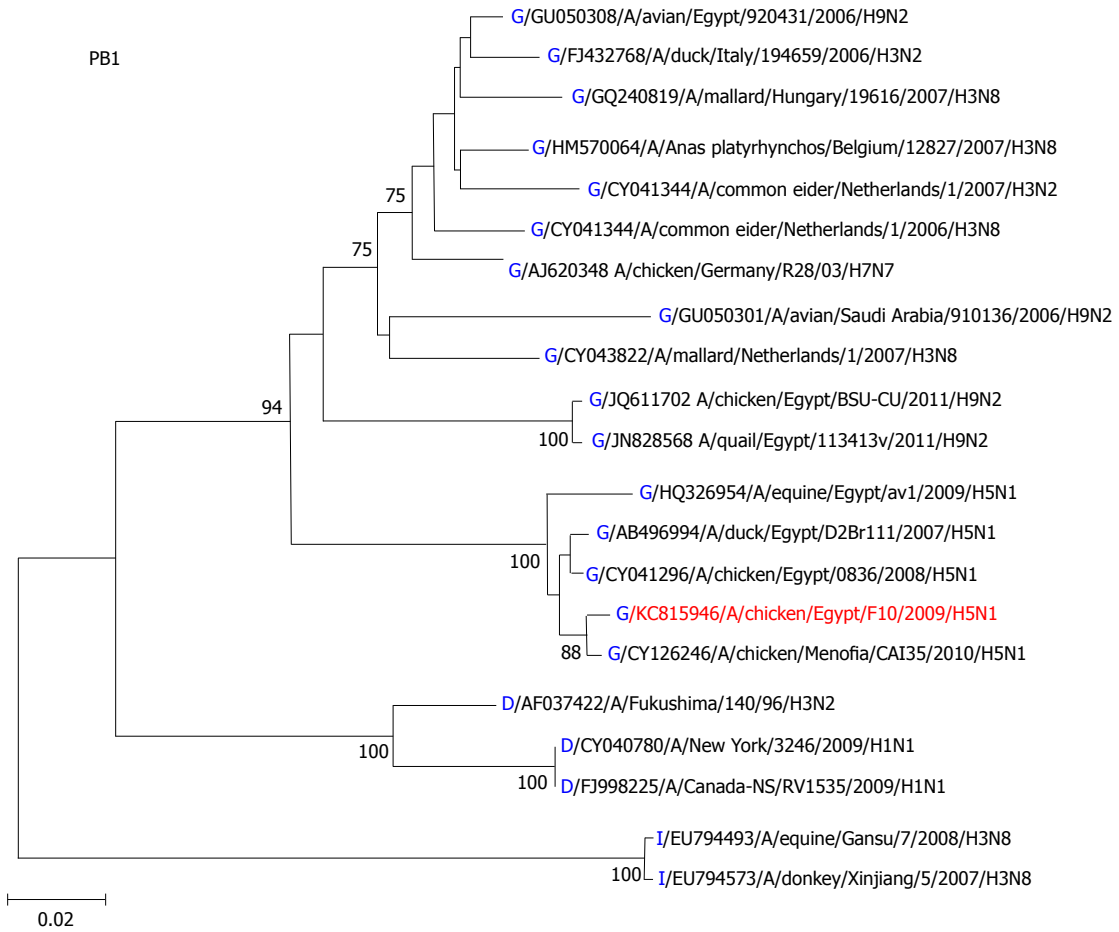
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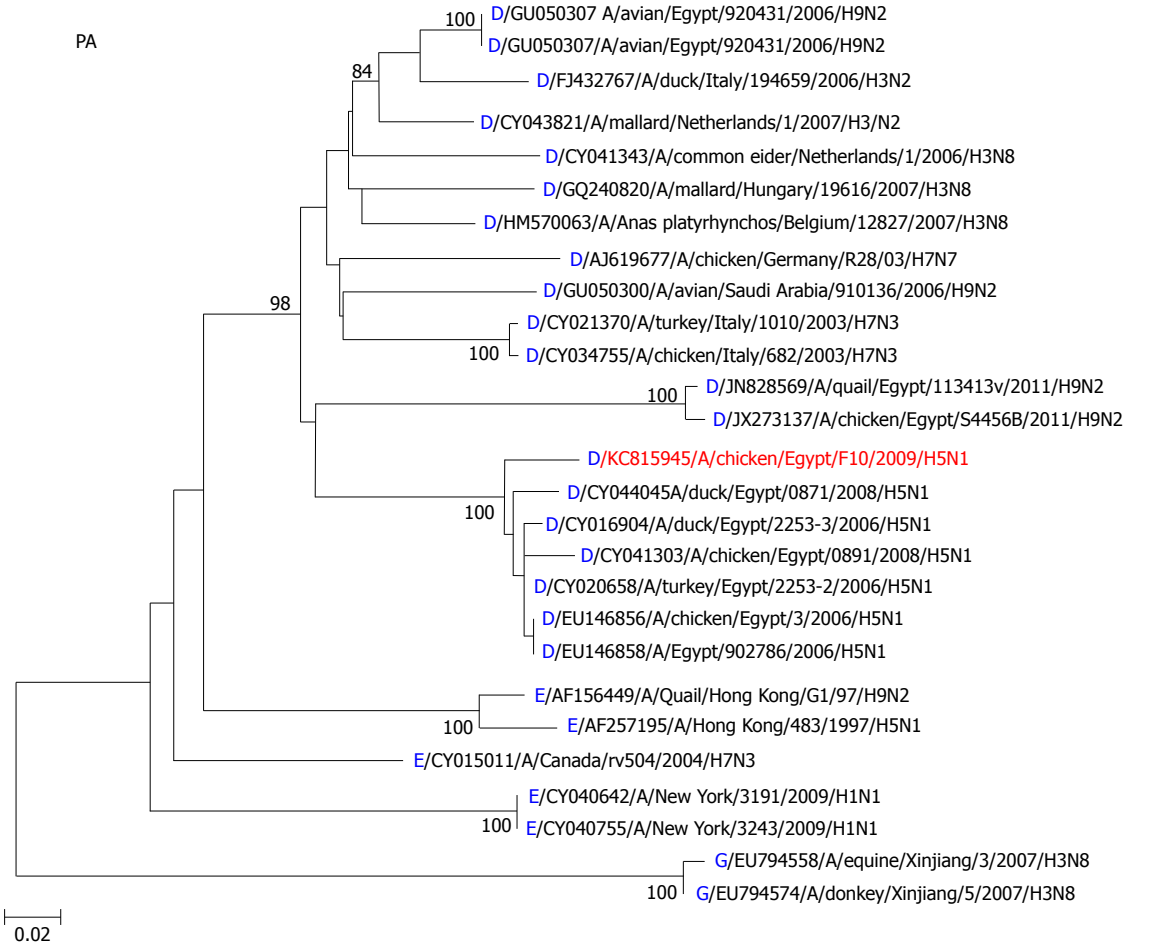
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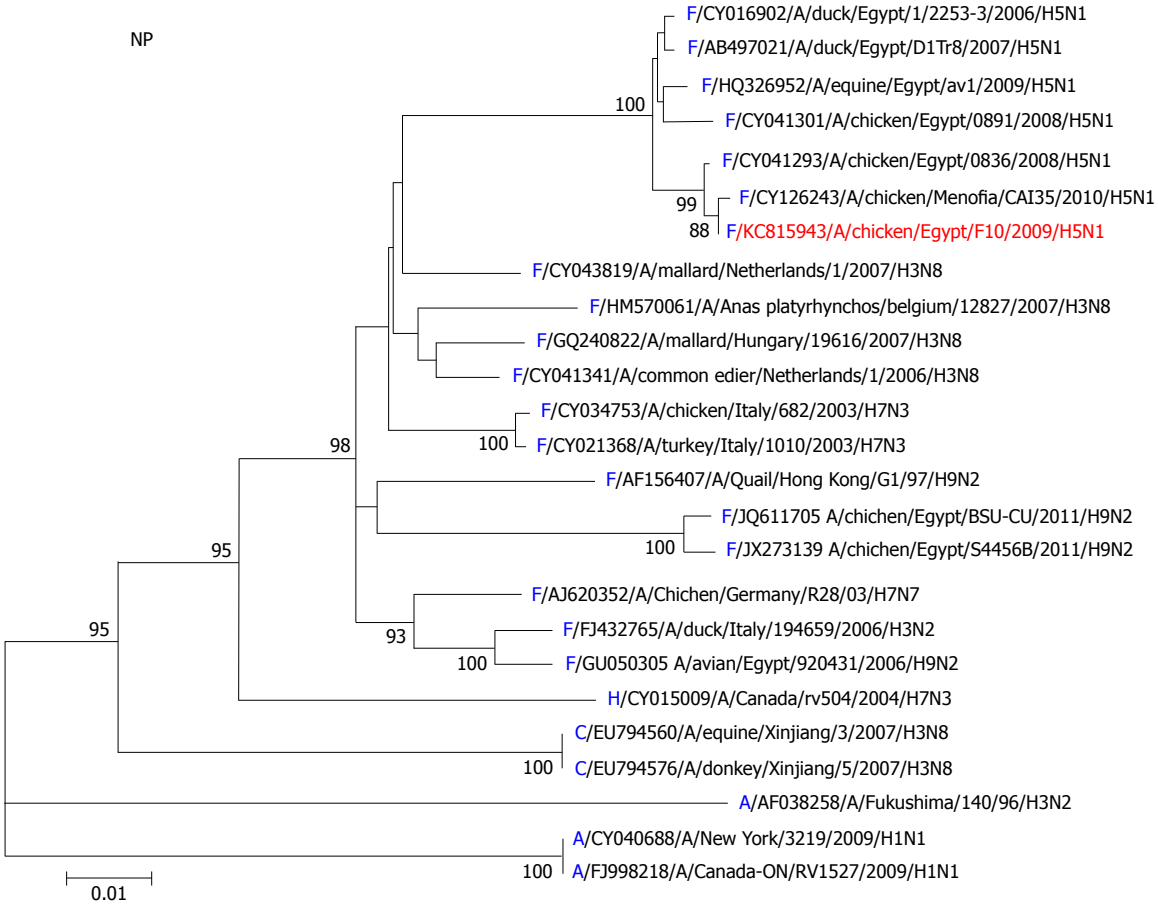
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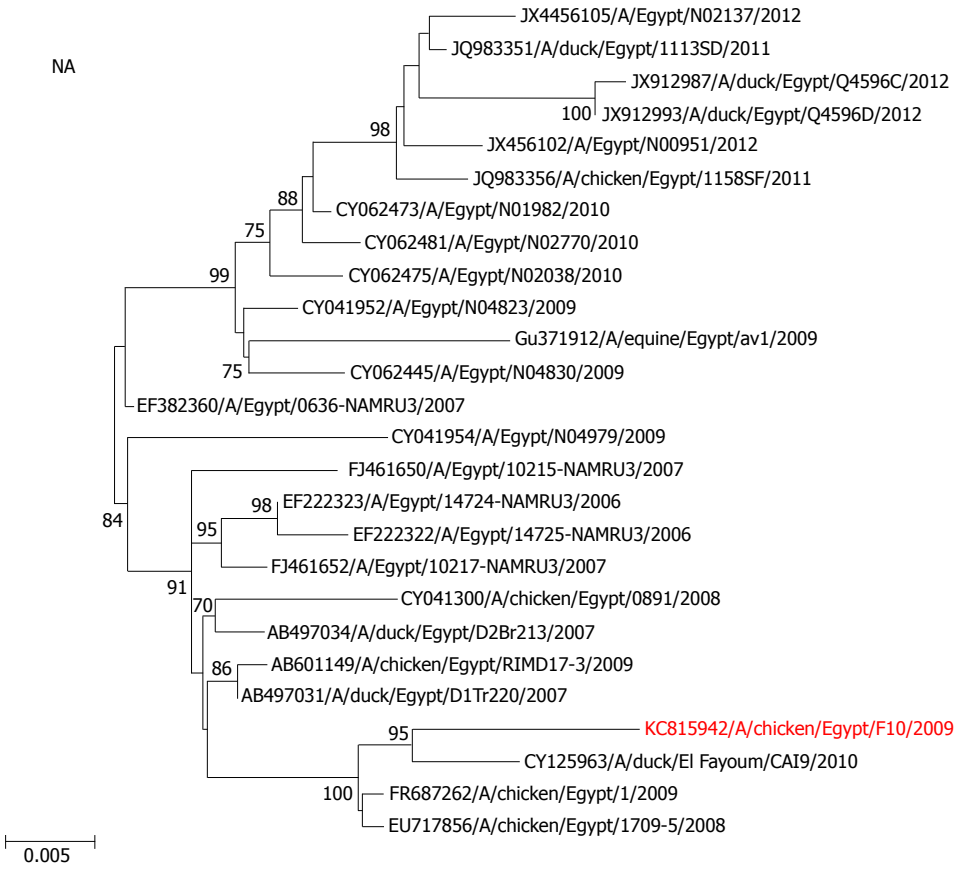
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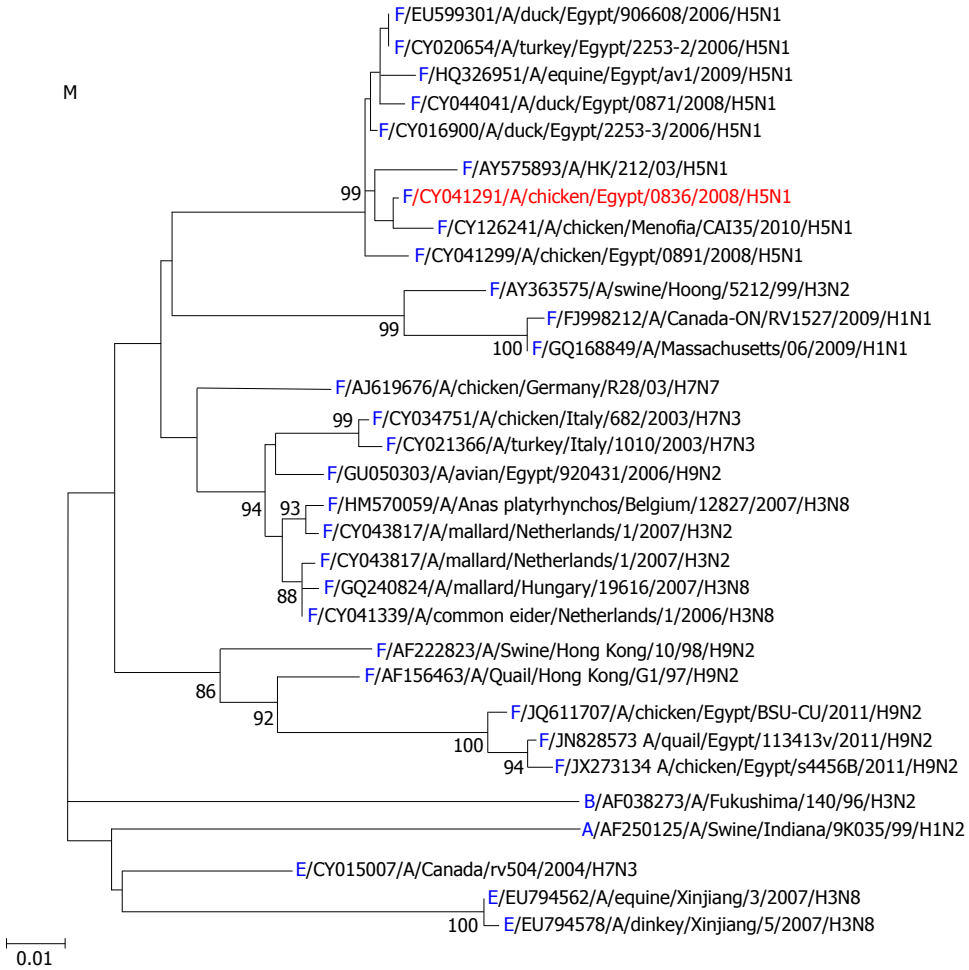
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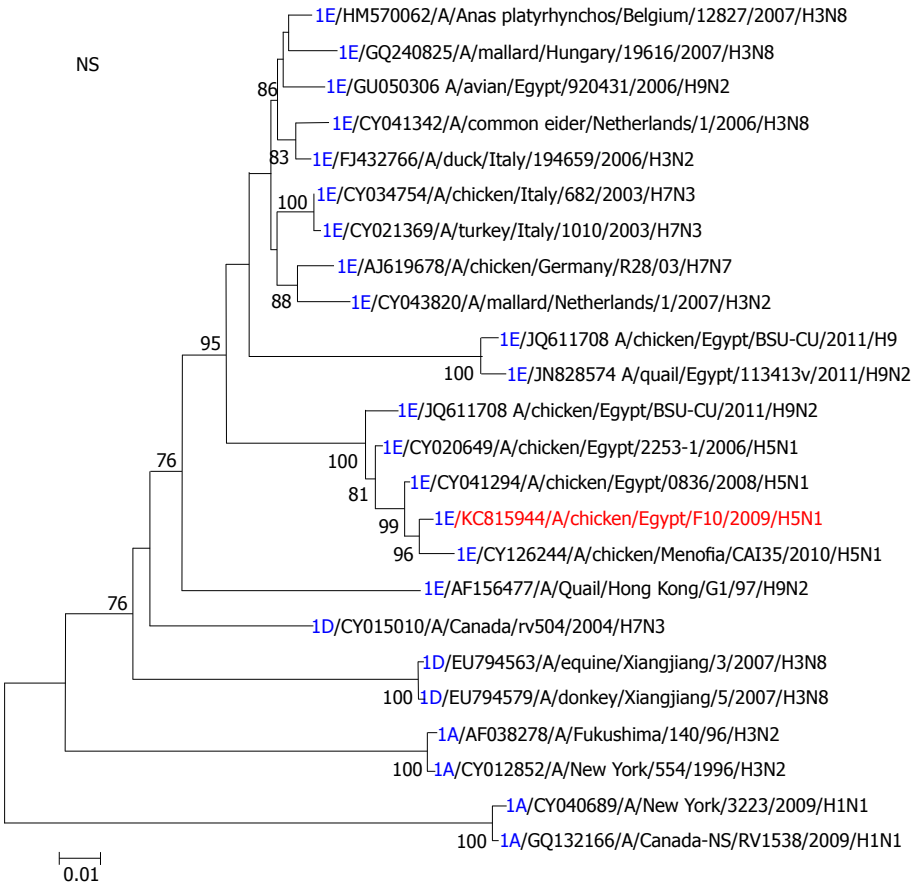

Figure 1 Phylogenetics of the viral genes of escape mutant H5N1 compared to selected influenza viruses. Escape mutant F10 strain examined in the current study was marked by a red color. The selected viruses were chosen to be representative from relevant sequences in GenBank database: H5N1 strains representative to the major gene lineages. Serotypes H1N1, H1N2, H3N2, H3N8, H7N3, H7N7 and H9N2 were also included in the phylogenetic trees of PB2, PB1, PA, NP, M and NS. For NA, Egyptian N1 sequences from Egyptian H5N1 strains were the only included sequences. The robustness of the individual nodes of the tree was assessed using a bootstrap of 1000 resembling in percent (70% and higher). Influenza A virus genotype tool (http://www.flugenome.org/ genotyping.php) was used to determine individual gene segment lineage. The genotype of each strain was mentioned in a blue color in the phylogenetic trees of PB2, PB1, PA, NP, M and NS.

in *PB2* (K627), *M2* (P69) and *NS1* (S42, E92/97). The mutation of aspartic acid (D) to glutamic acid (E) at position 92 (97 in strains with 5 amino acids deletion) was observed in this study in the F10 and also in other Egyptian H5N1 strains (Table 3). *PB2* of all Egyptian strains, including avian, mammalian isolates, possessed K627 (Table 3). F10 possessed virulent amino acid substitutions in *PB2* (K627), *M2* (P69) and *NS1* (S42, E97). All the detected virulent residues are also found in the other Egyptian H5N1 strains. Interestingly, all the Egyptian H5N1 strains possess virulent residue S64 in M2 protein while F10 possessed non virulent residue (L64) (Table 3). The *NS1* gene of F10 and other H5N1 Egyptian strains harbored L103F and I106M amino substitutions. The Egyptian H5N1 strains also possessed such amino acid substitutions (data not shown). Egyptian avian H5N1 strains including F10 possessed two human specific residues, E14 and R18 (data not shown).

DISCUSSION

Previous studies revealed that the *HA* genes from H5N1 Egyptian isolates were subjected to cumulative genetic drifts that resulted in further classification of the Egyptian strains into two sublineages [A(A1-A5) and B(B1-B5)][10]. A/chicken/Egypt/F10/2009 in the current study is related to the B2 sublineage. Eight amino acid substitutions were found in the Egyptian variants in lineage B, including the F10 strain at the amino acid positions P74S, D 97N, H110R, S123P, R140G, F144Y, N165H and A184E[10]. The deduced amino acid exchanges, as with most H5N1 Egyptian strains, showed polybasic cleavage motif consensus for clade 2.2 viruses, PGERRRKKR/GLF, while the consensus of 2.2, F10 PQGEGRRKKR/GLF, showed (R325G) substitution[10] with unknown significance. Lycett *et al*[17] specified 6 amino acid residues (86V, 124S, L/N138, T/S156, E/R212, T263) that are linked to the virulence of H5N1 in mammals. T156 and T263 were also present in F10 hemagglutinin[10].

In the current study, the different alleles of the F10 isolate were located within subtrees of the majority of the Egyptian strains. The influenza genotyping web tool revealed that the alleles of the F10, *PB2*, *PB1*, *PA*, *NP*, *NA*, *M* and *NS* alleles, are Eurasian in origin and related to K, G, D, F, 1J, F and 1E genotypes respectively[16].

Analysis of the *NA* gene revealed the presence of

the 20-amino acid deletion, a feature that is frequently observed during the process of adaptation of influenza viruses to poultry that are found to enhance the pathogenesis in chickens. The presence of amino acid arginine (R) at position 110 and the amino acid deletion in the *NA* are characteristic of clade 2.2 viruses[18]. The 228 (N to S) substitution is also present in the F10 and is an indication of 2.2.1 virus. Four *NA* mutations, E119G, H274Y, R292K and N294S, have been reported to confer resistance to *NA* inhibitors[19] but none were detected in the F10 isolate.

Chen *et al*[20] detected amino acid signatures specific to avian and human influenza A viruses. The six internal genes (*PB2*, *PB1*, *PA*, *NP*, *M* and *NS*) of A/chicken/Egypt/F10/2009 and most of Egyptian H5N1 strains showed avian like amino acid signatures.

Identification of the host range-specific amino acids could assume the functional sites that may mediate a host range. In a previous report, the amino acid sequences of the internal proteins in the Hong Kong poultry H5N1 viruses have been compared with those of other avian and human viruses[21]. The polymorphic amino acid residues in different protein sequences of the Egyptian human and avian strains, in comparison to the current escape mutant strain, were screened and the residues were classified as virulent or nonvirulent; such residues have functional significance for virulence in H5N1 to mammals[17]. Five virulent residues were detected in the avian H5N1 strains in *PB2* (K627), *M2* (S64, P69) and *NS1* (S42, E92/97); however, F10 showed only 4 virulent residues in *PB2* (K627), *M2* (P69) and *NS1* (S42, E92/97). An association between glutamic acid (E) at position 92 of the NS1 protein and resistance of H5N1 virus to interferons and TNF-α has been reported[22]. The mutation of aspartic acid (D) to glutamic acid (E) at position 92 (97 in strains with 5 amino acids deletion)[23] was observed in this study in the F10 and also in other Egyptian H5N1 strains. However, Seo *et al*[22] 2004 reported that this substitution possesses low impact in the virulence in mammals. E627K substitution in the PB2 protein is one of the genetic indicators for the adaptation and efficient replication in humans[24,25]. The temperature sensitivity of the virus and the efficacy of viral replication depend on the amino acid residue 627 of PB2. Viruses showing K627 displayed higher activity of the polymerase complex during viral replication at a lower temperature in comparison to viruses displaying E627[26]. Efficient virus replication may explain the wide host range of subtype H5N1 strains and their high virulence[26]. The PB2 of all Egyptian strains, including avian and mammalian isolates, possessed K627.

We have compared the amino acid residues associated with H5N1 virulence in mammals[11,17,23,25,27-31] to their corresponding residues in the A/chicken/Egypt/F10/2009. F10 possessed virulent amino acid substitutions in *PB2* (K627), *M2* (P69) and *NS1* (S42, E97). All the detected virulent residues are also found in the other Egyptian H5N1 strains. Interestingly, all the Egyptian H5N1 strains possess virulent residue S64 in the M2 protein, while F10 possessed non virulent residue (L64). P42S and D97E amino acid substitutions in the NS1 are responsible for the virulence of H5N1 in mammalian species and cytokine resistance[22]. In addition, amino acid substitutions L103F and I106M were found to be adaptive genetic determinants for growth and virulence in the NS1 gene of both mammals and avian[32]; F10 and other H5N1 Egyptian strain harbored these amino substitutions. The G184 that was detected in F10 and other H5N1 Egyptian strains contributes to the cleavage and the polyadenylation specificity factor binding and strongly affected the viral virulence[33].

Amantadine resistance is associated with one of the following M2 residues: 26, 27, 30, 31, 34, or 38[34,35]; however, the Egyptian H5N1 strains did possess such amino acid substitutions. Human, swine and avian specific M2 residues were determined[36]. Egyptian avian H5N1 strains, including F10, possessed two human specific residues, E14 and R18[36,37].

The genetic characteristics of the H5N1 virus isolates from chicken in Egypt provided evidence of a high possibility of inter-species transmission. The examined escape mutant H5N1 strain carried no clear amino acid signatures from other Egyptian H5N1 strains.

COMMENTS

Background

Avian influenza viruses showed considerable capacity to cross species barriers to infect susceptible mammals, including humans. Point mutations and reassortment possess a crucial effect on the virulence of HPAI H5N1. Escape mutants resulting from antigenic drift of the viruses were selected under vaccination. The current study aimed to investigate whether the escape mutant strain (A/chicken/Egypt/F10/2009) possesses reassortant genes or amino acid signatures that differentiate it from other classical strains.

Research frontiers

The high error-prone replication of influenza viruses and vaccination pressure unequivocally enhance the robustness of mutation capacity of the influenza viruses. The amino acid signatures of the escape mutant strains have not been addressed. In this study, the authors demonstrate the genetic constitution of the escape mutant strain and the possible amino acid signatures that could differentiate the escape mutant from other Egyptian H5N1.

Innovations and breakthroughs

Recent reports have highlighted critical amino acid substitutions in different alleles of influenza viruses that are associated with virulence to mammals. Amino acid signatures specific to avian and human influenza A viruses were also determined in previous reports. This study reported the presence of different amino acids substitutions in different alleles related to virulence to mammals; however, it failed to find the presence of prominent amino acid signatures in the examined escape mutant strain.

Applications

By understanding the amino acid substitutions in H5N1 escape mutants, its impact on virulence to mammals and how it could be accelerated under vaccination pressure, the avian influenza control procedure method based on vaccination should be reevaluated.

Terminology

Mutation at the *HA* epitope region is among the strategies the influenza virus uses to escape the immune system and represents the most important hindrance to vaccine development. Meanwhile, mutations in the other viral alleles play a crucial role in modulating virus pathogenicity to the original hosts and inter-species transmission to mammalian species, including humans.

Peer review

The authors studied the genetic constitution of the escape mutant H5N1 strain

in comparison with other influenza viral strains. Possible amino acid signatures were explored for identification of the escape mutant from other Egyptian H5N1 and different proteins with amino acid substitutions were also recorded compared to the available Egyptian H5N1 strains. The paper's scientific content is original and of good quality as a research article.

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