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Commentary

Nuclear trafficking of proteins from RNA viruses: Potential target for antivirals?

Leon Caly, Kylie M. Wagstaff, David A. Jans *

Department of Biochemistry and Molecular Biology, Monash University, Clayton 3800, VIC, Australia

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ABSTRACT

A key aspect of the infectious cycle of many viruses is the transport of specific viral proteins into the host cell nucleus to perturb the antiviral response. Examples include a number of RNA viruses that are significant human pathogens, such as human immunodeficiency virus (HIV)-1, influenza A, dengue, respiratory syncytial virus and rabies, as well agents that predominantly infect livestock, such as Rift valley fever virus and Venezuelan equine encephalitis virus. Inhibiting the nuclear trafficking of viral proteins as a therapeutic strategy offers an attractive possibility, with important recent progress having been made with respect to HIV-1 and dengue. The results validate nuclear protein import as an antiviral target, and suggest the identification and development of nuclear transport inhibitors as a viable therapeutic approach for a range of human and zoonotic pathogenic viruses.

Research over the last decade has established that a key part of the infectious cycle of many viruses involves the transport of specific viral proteins into the host cell nucleus, to perform specific functions relating to the modulation of transcription that are important for infection to proceed (Alvisi et al., 2007; Fulcher and Jans, 2011). This is particularly striking in the case of some RNA viruses in which replication occurs predominantly in the cytoplasm, but key proteins are transported to the nucleus. These include the nonstructural protein 5 (NS5) of dengue virus (DENV), which serves as the viral RNA polymerase; the matrix (M) protein of respiratory syncytial virus (RSV), which plays a key role in virion assembly; and the nonstructural "s" (NSs) protein of Rift valley fever (RVF) virus, which blocks interferon production (Bird et al., 2008; Ghildyal et al., 2009; Pryor et al., 2007) (see Table 1). As discussed below, these results implicate the transport of viral proteins into the nucleus as a promising target for therapeutic intervention.

Signal-dependent transport into and out of the eukaryotic cell nucleus occurs through nuclear pore complexes (NPCs) embedded in the nuclear envelope, which separates the nucleus from the cytoplasm (Fig. 1). Transport across the NPC is mediated by members of the importin (IMP) superfamily of transporters, of which there are multiple α and β subtypes. For transport into the nucleus, either a heterodimer of IMPα and β1 or one of the IMPβs, binds specifically to nuclear targeting sequences (nuclear localisation sequences or NLSs) within their respective cargo proteins, to mediate passage through the NPC into the nucleus (Fig. 1A left) (Alvisi et al., 2007; Fulcher and Jans, 2011). For transport out of the nucleus, cargo proteins bearing nuclear export sequences (NESs) are recognized by specific "exportin" (EXP) members of the IMPβ superfamily in order to enable export from the nucleus through the NPC (Fig. 1A right) (Kudo et al., 1997). EXP1 (Crm1) is the best characterized EXP, largely due to the fact that a specific inhibitor of cargo recognition, leptomycin B, (LMB), can be used in live, intact cells. However, even though agents such as LMB have been shown to inhibit viral infection, as in the case of RSV, nucleocytoplasmic trafficking of viral proteins has thus far been largely overlooked as a target for antiviral therapy (Ghildyal et al., 2009; Rawlinson et al., 2009).

A variety of drugs are now available that block the HIV-1 lifecycle at key stages, including cellular entry, reverse transcriptase and protease activity. However, the inherently high rate of emergence of drug-resistant HIV-1 strains highlights the need for alternative drug targets. Nuclear import offers an exciting possibility, since one of the key steps of the HIV-1 replication cycle is the transport of newly synthesized viral cDNA, surrounded by numerous viral and cellular proteins (the "pre-integration complex" (PIC)) into the nucleus (Fig. 1B left). Nuclear entry of the PIC is essential for integration of the HIV-1 genome into the host DNA, mediated by the HIV-1 integrase (IN) protein, without which replication cannot proceed. Interestingly, many of the proteins associated with the PIC are known to promote transport through the NPC, with the virally derived IN, matrix (MA) and Vpr (viral regulatory protein),

* Corresponding author. Tel.: +61 3 9902 9341; fax: +61 3 9902 9500.
E-mail address: david.jans@monash.edu (D.A. Jans).

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Table 1
Selected examples in which nuclear import or export of specific viral components is critical to infection.

Viral protein	IMP/EXP	Role in viral infection
Human immunodeficiency virus IN	IMPαβ1, IMP7, TNP03	Through interaction with IMPα/β1, and possibly through additional contributions from IMP7 and/or TNPO3, IN is a key mediator of transport of the HIV genome into the nucleus prior to integration. Inhibitors of IN–IMPα/β1 interaction reduce HIV infection 2–3-fold (Wagstaff et al., 2012)
Influenza virus PA, PB1, PB2 and NP proteins	IMPα, IMP5	The influenza RNP, comprised of the PA, PB1, PB2 and NP proteins and with the vRNA, is responsible for transcription of the negative sense vRNA in the host cell nucleus. vRNA–NP and PB2 localize independently in the nucleus through IMPα, while PA/PB1 heterodimers are imported through IMP5. Prevention of RNP interaction with either IMPα or IMP5 has been shown to reduce virus production (Cros et al., 2005; Hutchinson et al., 2011)
Dengue virus NS5	IMPα/β1, EXP1	Through interaction with IMPα/β1, NS5 localizes in the nucleus to impact on specific host gene transcription to facilitate DENV infection. Prevention of NS5–IMPα/β1 interaction by mutation reduces virus yield c. 1000 fold (Pryor et al., 2007), whilst inhibitors of NS5–IMPα/β1 (Wagstaff et al., 2012) interaction reduce virus production c. 6-fold. Prevention of NS5–EXP1 interaction by mutation abolishes virus production (Rawlinson et al., 2009), presumably by preventing replication of the DENV genome in the cytoplasm
Respiratory syncytial virus matrix	IMPβ1, EXP1	Through interaction with IMPβ1, M localizes in the nucleus to inhibit host-cell transcription generally and subvert the antiviral response. Prevention of M-IMPβ1 interaction by mutation reduces RSV virus production, while prevention of M-EXP1 interaction by mutation abolishes virus production (Ghildyal et al., 2009), presumably by preventing virus assembly in the cytoplasm
Rift valley fever virus NSs	??	NSs protein localizes within the infected cell nucleus to form ribbon-like structures that suppress transcription. Mutations that abrogate nuclear ribbon structure formation render RVFV avirulent (Bird et al., 2008; Le May et al., 2008)
Chicken anemia:anaemia virus VP3	IMPβ1, EXP1	Prevention of VP3 nuclear export by mutation results in a >20-fold reduction in CAV virus yield due to loss of viral particle formation (Prasetyo et al., 2009)
Venezuelan Equine encephalitis virus CA	IMPα/β1, EXP1	CA binds to both IMPα/β1 and EXP1 to form a tetrameric complex which obstructs the nuclear pore, blocking IFN-β production and the antiviral response (Atasheva et al., 2010). Mutations within CA that prevent IMPα/β1 and EXP1 interaction render VEEV less virulent
Human cytomegalovirus ppUL44	IMPα/β1	Nuclear localisation of the polymerase processivity factor ppUL44 is essential for viral replication. C-terminal truncation of ppUL44 to prevent IMPα/β1 binding reduces CMV virus yield up to 97% (Ripalti et al., 1995)

Abbreviations: IMP, importin; EXP, exportin; TNPO, transportin; IN, integrase; RNP, ribonucleoprotein; RdRp, RNA-dependent RNA polymerase; PA, polymerase acidic; PB1/2, protein basic 1/2 NP, nucleoprotein; NS5, nonstructural protein 5; NSs, nonstructural protein s; VP3, viral protein 3; CA, capsid; IFN, interferon.

and host cell LEDGF/p75 all identified as possessing potential NLSs, suggesting they play a role in PIC nuclear import (Bukrinsky et al., 1993; Fouchier et al., 1997; Heinzinger et al., 1994; Kootstra and Schuitemaker, 1999; Petit et al., 2000; Reil et al., 1998). A number of IMPs, including IMPα, IMPβ1, IMP7 and transportin 3 (TNPO3), have also been implicated (Ao et al., 2007,2010; Hearps and Jans, 2006; Levin et al., 2010).

These findings imply that HIV-1 makes use of multiple import pathways to ensure efficient delivery of its genome into the nucleus under diverse conditions and in diverse cell types, underlining the critical nature of this step in the replication cycle, and suggesting it as a target for therapeutic intervention (Piller et al., 2003). Recent analysis suggests that IN is a key nucleophilic component of the PIC, able to bind with high affinity not only to DNA, but also to specific IMPs (Hearps and Jans, 2006; Levin et al., 2010). Most anti-IN compounds have been developed to target the interaction of IN with DNA, and drug resistance is already emerging. Because IN must enter the nucleus before it can integrate the viral genome, it seems clear that abrogating nuclear import of IN is an attractive target for intervention (Fig. 1B left). Similarly, blocking PIC nuclear import by targeting IN is likely to be a rewarding approach to prevent infection (Lu et al., 2004).

The DNA binding domains of IN can tolerate mutations that result in a partial loss of activity, but confer drug resistance (Kuritzkes, 2011). Recent studies have focused on characterizing peptides that compete with the IN–NLS for IMPs (Levin et al., 2009) and on identifying small molecules that directly inhibit IN:IMPα/β1 interaction (Wagstaff et al., 2011). Both approaches have yielded agents that markedly reduce IN nuclear localization. To eliminate the potential host cell toxicity of substances that directly target IMPαβ1, Wagstaff et al. (2011) used a novel counterscreening approach to discard agents that inhibit binding to another well-characterized viral protein, simian virus SV40 large tumor antigen, enabling the identification of molecules that target the IN:IMPα/β1 interface. One of these compounds, mifepristone, specifically inhibited nuclear import of IN, but had no effect on a range of other viral and host cell proteins that localize in the nucleus through different IMP-dependent pathways, including those dependent on IMPα/β1 (Wagstaff et al., 2012). Considered counterscreening approaches can thus enable agents to be identified that are unlikely to cause toxicity, as they do not target host cell factors. Importantly, both specific and broad-spectrum compounds that block IN nuclear import can inhibit HIV-1 infection (Wagstaff et al., 2012), validating the inhibition of nuclear import as an antiviral strategy. Because the toxic effects of compounds targeting host cell IMPs is likely to prevent their use in a clinical context, as shown conclusively for LMB (elactocin) and EXP1 (Newlands et al., 1996), these studies pave the way for new approaches to identify novel agents that specifically target virus:host cell interfaces (see also (Loregian et al., 2002)).

Influenza virus also offers targets for inhibition of nuclear transport. In analogous fashion to the HIV-1 PIC, its ribonucleoprotein (RNP) is a multi-protein nucleic acid (RNA) complex which must be present in the nucleus for productive infection. The key components are the vRNA binding NP (nucleoprotein) and the viral RNA-dependent RNA polymerase (vRdRp), composed of the proteins PB1, PB2 (protein basic 1 and 2) and PA (polymerase). Nuclear import of the vRNA through the action of NP is dependent on interactions with IMPα (Wu et al., 2007), but PB1/PA and PB2 make use of IMP5 and IMPα, respectively, for their transport into the nucleus (Boivin and Hart, 2011; Deng et al., 2006; Hutchinson et al., 2011). Preliminary studies have shown that attenuating the interaction of PB1 with IMP5, or of NP with IMPα, through mutation or competition approaches, reduces viral yield (Cros et al., 2005; Hutchinson et al., 2011), presumably through a lack of vRNA transcription and viral mRNA production within the nucleus. siRNA knockdown of IMPα or β1 also inhibits viral replication (Zhong et al., 2012), supporting the idea that nuclear import of the NP complex is a viable therapeutic target.

No drugs are currently available for of the treatment of dengue fever and its severe variant, dengue hemorrhagic fever, but targeting nuclear import offers a potential antiviral strategy. DENV replication occurs in the cytoplasm, with no requirement for its genome to enter the nucleus (Bartenschlager and Miller, 2008;

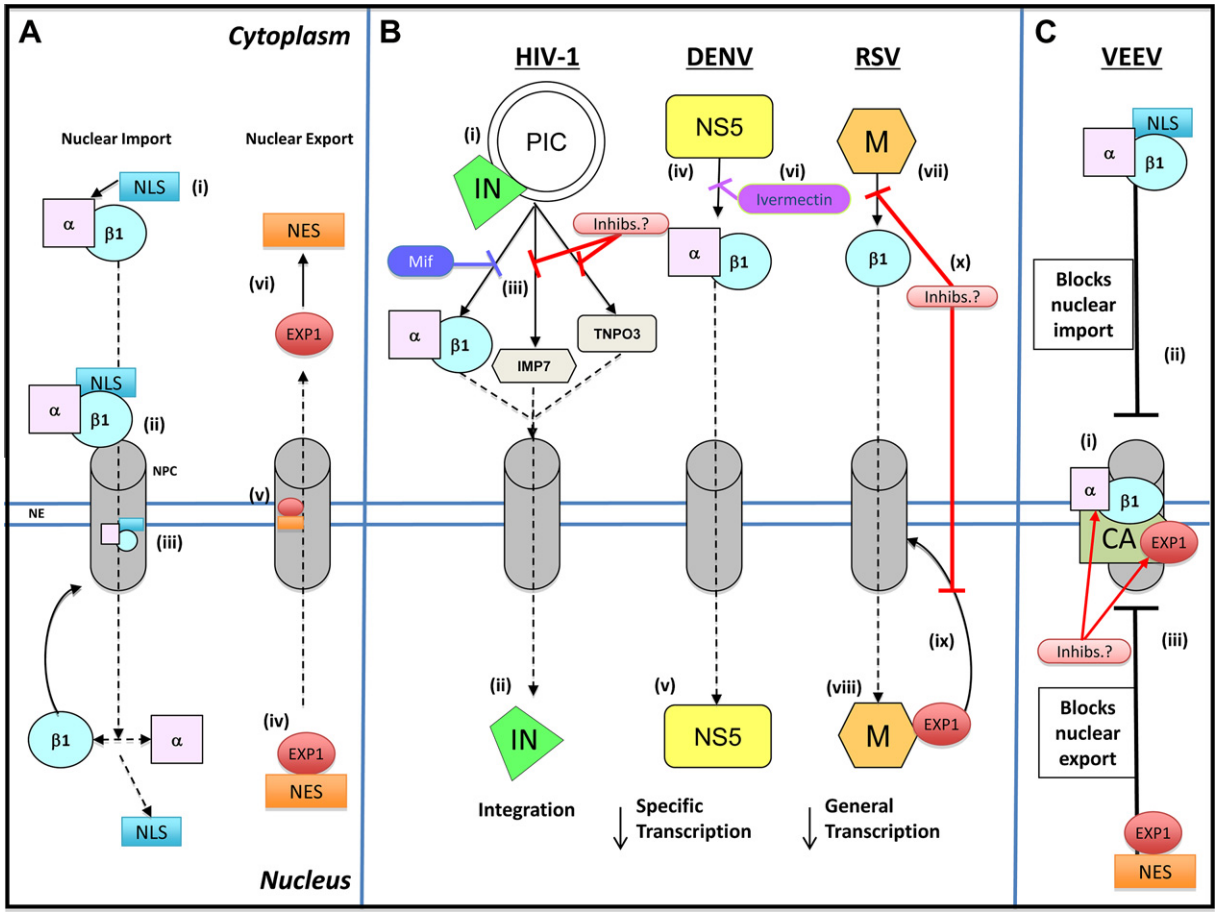

Fig. 1. Targeting viral protein interaction with the cellular nuclear transport machinery. A. Classical nuclear protein import requires (i) recognition of nuclear localisation signal (NLS)-containing cargo proteins, either by the IMPα/β heterodimer or by IMPβ1 or homologues thereof (not shown), on the cytoplasmic side of the nuclear pore complex (NPC). (ii) The transport complex docks to the NPC and undergoes translocation to the nucleus through the central pore, via transient interactions with the nucleoporin proteins that comprise the NPC. (iii) Once within the nucleus, the transport complex dissociates to allow the cargo to perform its nuclear function. Nuclear export (iv) involves allows recognition of leucine-rich nuclear export signal (NES)-containing cargo proteins by EXP1, and transport through the pore via transient interactions between EXP1 and the nucleoporins of the NPC (v), followed by dissociation within the cytoplasm (vi). B. Nuclear import and/or export of key components is a feature of many viruses, including HIV-1, DENV and RSV. An example is the HIV-1 IN protein, which alone or as part of the HIV-1 pre-integration complex (PIC), is transported into the nucleus, dependent on interaction with (i) IMPs α/β1, 7 or TNPO3, after which IN integrates the viral cDNA into the host cell genome (ii), a prerequisite for productive infection. Inhibitors (Inhibs.), such as mifepristone (Mif), which can specifically block IN:IMPαβ1 interaction (iii), or those targeting other IMPs implicated in PIC nuclear import (Ao et al., 2007,2010; Hearps and Jans, 2006; Levin et al., 2010) can be used to inhibit nuclear import of IN and/or the PIC to prevent integration. In comparable fashion (iv), DENV NS5 depends on interaction with IMPα/β_1 to traffic to the nucleus, where it acts as a specific transcriptional modulator (e.g., repressor of IL-8 expression) (v). Blocking NS5 nuclear import with an inhibitor such as ivermectin (vi) may represent an attractive target for therapeutic intervention. Analogously, (vii), the RSV M protein depends on interaction with IMPβ1 early in infection to localize in the nucleus, where it suppresses general host-cell transcription (viii). Later in infection (ix), M is exported to the cytoplasm to play a key role in pro-virion assembly. Inhibitors of interaction of M with IMPβ1 and its nuclear export receptor EXP1 (x) represent potential anti-virals to combat RSV. C. During infection, the VEEV CA protein binds simultaneously to the IMPα/β heterodimer and to EXP1 (i), forming a large tetrameric complex which blocks the NPC, preventing all transport into and out of the nucleus (ii) and suppressing STAT1-mediated antiviral responses (Atasheva et al., 2010). Blocking binding of CA to IMPαβ and/or EXP1 represents an attractive target to inhibit infection.

den Boon et al., 2010). However, the NS5 protein, which plays a central role in replication (Davidson, 2009; Koonin, 1993) is predominantly found within the nucleus of infected cells (Pryor et al., 2007; Rawlinson et al., 2006), as a result of specific interaction with the IMPα/β1 heterodimer (see Fig. 1B middle). One role of NS5 in the nucleus appears to be to repress the specific transcription of genes important in the host antiviral response, such as interleukin-8 (Pryor et al., 2007; Rawlinson et al., 2009). Impairing the nuclear import of NS5, through mutation of its IMPα/β1-recognized NLS, results in reduced viral viability, with a corresponding increase in IL-8 levels (Pryor et al., 2007). Importantly, inhibiting NS5 nuclear import using ivermectin, a general inhibitor of IMPα/β1-dependent nuclear import, greatly reduces virus production (Wagstaff et al., 2012), providing proof of principle that inhibitors of nuclear import represent viable anti-DENV agents.

Although RSV is a typical paramyxovirus that replicates in the cytosol, its M protein is transported into the nucleus by IMPβ1 early in infection, where it inhibits general host cell transcription through an as-yet unknown mechanism (Fig. 1B right) (Ghildyal et al., 2003, 2005a). Later in infection, M is returned to the cytoplasm by EXP1 (Ghildyal et al., 2009), where it functions as the key viral assembly factor, acting as an adaptor to bring together newly-formed nucleocapsids and envelope F and G glycoproteins (Ghildyal et al., 2002, 2005b, 2006). Although RSV replication and assembly occur within the cytoplasm, mutations within the IMPβ1-recognized NLS of the M protein result in a 20-fold reduction in virus yield (Ghildyal et al., 2009), implying that nuclear import represents a viable target for the development of RSV-specific antiviral agents. Analogously, M nuclear export through EXP1 would also appear to be an interesting target, based on the fact that viruses with mutations in the EXP1-recognized NES of M are not viable, presumably due to the critical requirement for M in the cytoplasm during virion assembly (Ghildyal et al., 2009). Nucleocytoplasmic trafficking of RSV M therefore represents a target as

exciting as the nuclear import of HIV-1 IN or DENV NS5 for future drug development.

Nuclear trafficking is also critical for the replication of a number of viruses that have far-reaching economic consequences with respect to livestock, such as the bunyavirus RVFV, which in addition to substantial numbers of human victims in sub-Saharan Africa, is responsible for the loss of many thousands of animals. RVFV replicates within the cytoplasm of infected cells, but its NSs protein traffics to the nucleus, where it forms unique ribbon-like structures (Struthers et al., 1984). Within these structures, NSs interacts with and sequesters numerous cellular factors, including SAP30, which is involved in regulating IFN-β expression, and p44, a component of the TFIIH transcriptional complex, resulting in a global reduction in host cell transcription (Kalveram et al., 2011; Le May et al., 2004). Nuclear localisation of NSs, and its ability to interact with SAP30, are potentially the key pathogenic factors/steps in RVFV infection, since the SAP30-binding mutant NSsΔ210–230 is no longer virulent for mice (Le May et al., 2008). Elucidating the nuclear import pathway of NSs will be an important first step towards developing inhibitors that may block the effects of RVFV on host cell transcription.

Another threat to both humans and animals, Venezuelan equine encephalitis virus (VEEV) suppresses host-cell antiviral responses through transcriptional repression mediated by its capsid (CA) protein, which has the unique ability to block transport into and out of the nucleus through the NPC (Fig. 1C). This block is believed to be mediated by a unique 39-amino acid sequence within the CA protein that binds simultaneously to IMPαβ1 and EXP1, forming a large tetrameric complex that occludes the NPC, preventing all transport through it, including both the nuclear import of transcription factors such as signal transducers and activators of transcription (STATs) required for an antiviral response, and the export of newly synthesized mRNA (Atasheva et al., 2010). Some non-pathogenic VEEV derivatives have mutations within this amino acid sequence, supporting the idea that CA is a key pathogenic factor (Atasheva et al., 2010; Frolov et al., 2012). Molecules that specifically inhibit capsid-IMP/EXP binding, thus permitting nuclear trafficking and a robust antiviral response, represent an exciting prospect as the basis of future therapies.

1. Future perspectives

Many RNA viruses rely on nucleocytoplasmic trafficking of specific viral gene products for efficient infection, validating nuclear transport as a target for the development of new antiviral drugs. However, although blocking the host cell nuclear transport machinery – whether import or export – can inhibit virus production, it is essential to recognize that targeting host cell proteins is inextricably intertwined with issues of toxicity (Newlands et al., 1996). Targeting the interfaces between viral and host proteins (e.g. importin-viral protein or exportin-viral protein interactions) appears to be the most promising strategy to avoid toxicity, as well as limiting the possibility that viral gene products will be able to mutate to eliminate drug binding, but still maintain functionality. Nucleocytoplasmic transport represents an exciting avenue for the future development of novel antiviral therapies directed at virus–host interfaces.

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