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Review

HIV-1 uncoating: connection to nuclear entry and regulation by host proteins

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ABSTRACT

The RNA genome of human immunodeficiency virus type 1 (HIV-1) is enclosed by a capsid shell that dissociates within the cell in a multistep process known as uncoating, which influences completion of reverse transcription of the viral genome. Double-stranded viral DNA is imported into the nucleus for integration into the host genome, a hallmark of retroviral infection. Reverse transcription, nuclear entry, and integration are coordinated by a capsid uncoating process that is regulated by cellular proteins. Although uncoating is not well understood, recent studies have revealed insights into the process, particularly with respect to nuclear import pathways and protection of the viral genome from DNA sensors. Understanding uncoating will be valuable toward developing novel antiretroviral therapies for HIV-infected individuals.

Contents

Introduction

As a retrovirus, HIV-1 infection requires reverse transcription of its single-stranded RNA genome into double-stranded DNA that is translocated into the nucleus and integrated into host cell chromatin. Cellular transcription results in synthesis of viral genomic

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RNA and proteins that assemble at the host cell plasma membrane for release as virions. Before these events can proceed, disassembly of a protective, conical capsid around the HIV-1 genome occurs after virus entry into the cell, in a process known as uncoating.

Uncoating of the HIV-1 core is highly regulated and plays a critical role during early post-entry stages of infection. The core consists of a conical viral capsid composed of a polymer of capsid protein (CA) subunits encasing the viral RNA genome and associated proteins, including nucleocapsid (NC), reverse transcriptase (RT), and integrase (IN). While still not completely understood, uncoating is likely a multistep process that begins with loosening or a small opening of the capsid, followed by stripping of most or all CA monomers from the core prior to entry into the nucleus. Perturbation of uncoating has detrimental effects on downstream replication steps and, ultimately, infectivity. For example, CA mutations that alter the intrinsic stability of the viral capsid lead to significant reduction in reverse transcription, trafficking of viral DNA to the nucleus, and infectivity. Because of the highly ordered nature of capsid dissociation required for viral infectivity and the unique structure of HIV-1 mature capsid that is intolerant of mutations, uncoating is a favorable target for antiretroviral therapy.

Complicating matters, however, the mechanism of uncoating likely requires several host cell proteins and trafficking pathways and is difficult to study *in vivo*. Uncoating appears to be influenced by cell type-specific factors and may vary in different cells. Our understanding of HIV-1 uncoating results mostly from studies of viral mutants and host factors, together with biochemical characterization of HIV-1 cores *in vitro*.

Structure of the HIV-1 core

The HIV-1 genome, like that of all retroviruses, encodes the Gag polyprotein that has three major domains: matrix (MA), CA, and NC. Gag oligomerizes in an infected cell at the plasma membrane for assembly and release into immature virions that have a spherical shaped core associated with the viral membrane (Fuller et al., 1997). Cleavage of Gag by HIV-1 protease leads to release of the each of the three mature proteins and further assembly into the mature core, which, unlike many retroviruses, is conical in shape. Although immature HIV-1 particles contain approximately 5000 Gag molecules, only 1000–1,500 CA molecules assemble into the mature capsid that encases the two packaged copies of the viral RNA genome (Briggs et al., 2004).

HIV-1 CA is a two-domain protein consisting primarily of alpha helices. Recombinant CA and CA-NC proteins can assemble *in vitro* into tubes and spheres, some of which resemble mature, conical cores (Campbell and Vogt, 1995; Ehrlich et al., 1992). The HIV-1 capsid was originally modeled as a lattice of CA hexamers that is closed by the insertion of 12 pentamers (Ganser et al., 1999). Two structures obtained from cryo-electron microscopy, and confirmed by crystallography, of *in vitro* assembled HIV-1 CA showed hexamers that are stabilized by an inner ring of six amino-terminal domains (NTDs) and an outer "girdle" of carboxyl-terminal domains (CTDs) that also form intersubunit contacts with adjacent NTDs. (Ganser-Pornillos et al., 2007; Li et al., 2000; Pornillos et al., 2009). The CTD also forms dimeric and trimeric interfaces connecting the hexamers. The native viral capsid is continuously curved, likely as a result of the flexibility of CTD dimers, particularly in helices 9 and 10 (Byeon et al., 2009; Zhao et al., 2013).

As mentioned above, the CA CTD also forms a trimer interface between hexamers, in which helix 10 of one hexamer interacts with helix 11 from an adjacent hexamer; this interface also plays a role in disassembly of the core (uncoating) in target cells (Byeon et al., 2009). Several amino acid substitutions in these helices destabilize or hyperstabilize cores, leading to loss of viral infectivity (Byeon et al., 2009; Forshey et al., 2002; von Schwedler et al., 2003; Zhao et al., 2013). Moreover, inter-hexamer cross-linking of introduced cysteine residues within the trimer interface resulted in resistance to *in vitro* disruption mediated by rhesus macaque TRIM5α (rhTRIM5α), which is discussed in more detail below (Zhao et al., 2011).

Experimental approaches to study uncoating

HIV-1 uncoating has been challenging to study owing to a lack of specific and sensitive assays to measure or visualize this process. In addition, cores from viruses and *in vitro* assembled structures are heterogeneous and many are defective, making it difficult to examine individual cores. Over the years, several *in vivo* and *in vitro* approaches have been used to study this process indirectly and are described here.

RTC/PIC isolation and biochemical characterization

Several groups have employed biochemical assays to isolate and characterize reverse transcription complexes (RTCs) or pre-integration complexes (PICs) recovered from the cytoplasm of freshly-infected cells (Bukrinsky et al., 1993; Farnet and Haseltine, 1991; Fassati and Goff, 2001; Gendelman et al., 1990; Karageorgos et al., 1993; Miller et al., 1997). Cytoplasmic extracts from HIV-infected cells, obtained by gentle detergent treatment or hypotonic lysis, are fractionated over sucrose gradients by ultracentrifugation. Fractions containing viral DNA sequences, which are present during and upon completion of reverse transcription, can be evaluated by multiple biochemical assays for the presence of viral and host proteins, viral nucleic acids, and functional assays. *In vitro* reverse transcription and integration assays can be performed with RTCs/PICs isolated under different conditions or in the presence or absence of cellular factors.

Proteins detected in association with viral DNA include MA, IN, RT, and Vpr, whereas little or no detectable CA remains. Differences in protein content observed by different researchers could be due to the timing of sample collection after infection and variations in the methods used to extract cytoplasmic fractions and to perform sedimentation. The early observation that these complexes generally lack CA suggested that uncoating occurs rapidly in target cells. However, HIV-1 capsid might be fragile and lost from the RTC/PIC during the isolation procedures. Moreover, as discussed below, subsequent studies also demonstrated a critical requirement for capsid stability in reverse transcription, arguing that uncoating is completed later in the life cycle. Nonetheless, the timing of uncoating, and whether it is progressive or cooperative, are questions yet to be definitively answered.

In vitro capsid stability and disassembly assay

HIV-1 cores can be isolated from viruses and assayed for disassembly *in vitro* (Forshey et al., 2002; Shah and Aiken, 2011). The process involves overlaying HIV-1 virions onto a layer of detergent atop a sucrose gradient. During ultracentrifugation at 4 °C, the viral membrane is removed through the detergent and multiple fractions are obtained throughout the gradient. Fractions that contain pelletable CA are characterized as lacking the envelope proteins gp41 and gp120 but containing viral RNA, RT, and IN (Forshey and Aiken, 2003; Forshey et al., 2002). Dissociation of CA from the purified cores can be measured after incubation at 37 °C and ultracentrifugation. Intact cores will be pelletable, whereas disassembled cores result in dissolution of CA. Similar to isolated

RTCs/PICs, reverse transcription can be assayed with cores isolated from virions under various conditions (Warrilow et al., 2008).

Viral genetics: mutations and chimeric viruses

Several research groups noticed that mutations in the CA region of Gag influence early events in the HIV-1 life cycle, including initiation or completion of reverse transcription (Reicin et al., 1996; Tang et al., 2001). Likewise, *in vitro* core stability can be altered by introduction of CA mutations, leading to unstable or hyperstable capsids and loss of virus infectivity (Forshey et al., 2002). Characterization of specific mutants can reveal important sites for binding host cell factors or alterations in the capsid structure, both of which may provide insight into uncoating mechanisms (Abdurahman et al., 2007; Brun et al., 2008; Dismuke and Aiken, 2006; Fitzon et al., 2000; Li et al., 2009; Misumi et al., 2010; Noviello et al., 2011; Qi et al., 2008; Scholz et al., 2005; Tang et al., 2001, 2003, 2007; von Schwedler et al., 2003; Wacharapornin et al., 2007; Yang et al., 2012; Yufenyuy and Aiken, 2013).

Another experimental approach involves replacement of HIV-1 CA with CA from other retroviruses, such as murine leukemia virus (MLV) or simian immunodeficiency virus (SIV). Studies of such chimeric viruses have revealed virus–host interactions involved in infection of specific cell types, including simian cells and non-dividing cells (Cowan et al., 2002; Fujita et al., 2001; Hatziioannou et al., 2006; Yamashita and Emerman, 2004). Comparison to wild-type HIV-1 and other retroviruses provides insight into species-specific factors that influence HIV-1 post-entry steps as well as structural differences that affect uncoating.

Cellular genetics: protein knockdowns and expression of restriction factors

Small interfering RNA (siRNA) has been employed in numerous studies to determine whether specific host cell proteins control the early steps of HIV-1 infection. Most notably, in 2008 three groups screened siRNA libraries to identify multiple proteins involved in early HIV-1 life cycle events (Brass et al., 2008; Konig et al., 2008; Zhou et al., 2008). Further characterization of several of the knockdowns, discussed later, showed that the corresponding host proteins bind to HIV-1 CA and are required for proper reverse transcription and nuclear entry.

Conversely, factors thought to influence uncoating can be expressed in cells to study the kinetics or inhibition of HIV-1 uncoating. The HIV-1 capsid is targeted by primate-specific restriction factors. Two nonhuman primate proteins, rhTRIM5α and an owl monkey fusion of TRIM5α and cyclophilin A (CypA), TRIMCyp, bind to the capsid and inhibit infection when expressed in cells (Ganser-Pornillos et al., 2011; Sayah et al., 2004; Stremlau et al., 2004; Yap et al., 2004). These factors are partly responsible for the inability of HIV-1 to replicate inefficiently in the cells of many nonhuman primates. Like unstable mutant CA cores, these restriction factors lead to premature uncoating of HIV-1 when expressed in target cells, and can disrupt capsid-like CA assemblies *in vitro* (Black and Aiken, 2010; Chatterji et al., 2006; Diaz-Griffero et al., 2007; Langelier et al., 2008; Stremlau et al., 2006; Zhao et al., 2011). Induction of premature uncoating by rhTRIM5α is also associated with inhibition of reverse transcription (Stremlau et al., 2004; Yap et al., 2006) and requires proteasome activity in target cells (Anderson et al., 2006; Kutluay et al., 2013; Wu et al., 2006). These effects are abolished by specific mutations in the TRIM5α RING domain, implicating ubiquitin ligase activity in the mechanism (Roa et al., 2012).

Pharmacological approaches

Several small molecule HIV-1 inhibitors target CA, with many of these disrupting immature particle assembly or maturation, late steps in the viral life cycle (Lemke et al., 2012; Li et al., 2003; Tang et al., 2003; Ternois et al., 2005; Zhang et al., 2008). However, some CA-binding compounds have been identified that act during post-entry steps of infection, which would suggest that they perturb uncoating. The first compound in this class, PF-3450074 (PF74), was shown to destabilize HIV-1 capsid after entry into cells (Blair et al., 2010; Shi et al., 2011). Crystallization of PF74 with the N-terminal domain of CA suggested that the compound binds in a pocket formed by helices 3, 4, 5, and 7 (Blair et al., 2010), which is distinct from the binding site of the late stage inhibitors mentioned above. PF74 stimulated the uncoating of purified HIV-1 cores *in vitro*, and decreased the recovery of pelletable CA upon lysis of target cells, suggesting that the compound induces premature uncoating. An HIV-1 mutant selected for PF74 resistance acquired mutations in helices 4 and 5 (Q67H, K70R, T107N, L111I) and in the CypA binding loop (H87P). These changes collectively conferred strong resistance to the inhibitor by reducing binding (Blair et al., 2010; Shi et al., 2011). They also stabilized the viral capsid *in vitro* and delayed its opening in the target cell (Xu et al., 2013). Interestingly, two other molecules, BI-1 and BI-2, were reported to bind at the same CA site as PF74 but stabilized *in vitro* CA-NC tubes and inhibited HIV-1 infection (Lamorte et al., 2013). Structural analysis showed that the BI compounds interact with the same site as PF74, but occupy a smaller volume within the pocket. Another small molecule, and its derivative I-XW-053, were designed to bind at a novel site at the NTD–NTD interface, interacting with residues I37 in helix 2 and R173 in helix 8 (Kortagere et al., 2012). Like PF74, this compound also reduced early reverse transcripts, suggesting that uncoating was altered. Collectively, these studies demonstrate that the HIV-1 capsid is a viable target for antiviral development.

Imaging assays

Visualization of HIV-1 uncoating *in vivo* using fluorescence or electron microscopy techniques is challenging, mainly due to the dynamic process itself and inefficient labeling of viral proteins or genomes during infection. While some viral PIC components, such as Vpr and IN, can be tagged without apparent loss of function (Albanese et al., 2008; Arhel et al., 2006; McDonald et al., 2002), introduction of fluorophores on CA without disruption of proteolytic processing and proper assembly into cores has proven to be technically challenging. Phenotypic mixing of WT CA and CA containing a small tetracysteine (TC) that can be labeled by a fluorescent dye can generate particles suitable for imaging studies (Campbell et al., 2008). TC-tagged CA was shown to co-localize with GFP-tagged rhTRIM5α and mCherry-Vpr. To monitor reverse transcription, fluorescently tagged deoxynucleotides (dNTPs) were microinjected into cells and were co-localized with tagged-Vpr after infection (McDonald et al., 2002), but the background and technical challenges of this procedure make it impractical. Antibody staining of fixed, HIV-infected cells showed that CA is co-localized with fluorescently labeled Vpr, IN, dNTPs, or viral RNA, but co-localization was rare and the procedure cannot be used for continuous monitoring during infection (Albanese et al., 2008; Arhel et al., 2006, 2007; Campbell et al., 2008; Hulme et al., 2011; McDonald et al., 2002; Xu et al., 2013). However, a recent study employed fluorescently tagged APOBEC3F that was incorporated into virions and remained associated with RTCs and PICs following entry into the nucleus (Burdick et al., 2013), which may permit tracking of these complexes in real time. Additionally, our group has shown that modified uridines, which can react with a specific

fluorescent dye, can be introduced into the viral RNA genome (Xu et al., 2013). The dye appeared to access the viral RNA only after opening of the capsid *in vivo* or in the presence of cellular factors *in vitro*, thus providing a novel probe for genome accessibility during the early steps of HIV-1 infection.

Electron microscopy has revealed intriguing higher resolution viral structures *in vivo*. Correlative 3-dimensional live-cell and electron microscopy revealed RTCs by fluorescent Vpr and dNTPs (McDonald et al., 2002). Also using correlative cryo-EM using Vpr-labeled virions, intact HIV-1 capsids could be visualized in the cytoplasm of infected cells (Jun et al., 2011). Gold labeling of anti-CA antibodies also revealed CA particles in infected T cell lines near the nuclear pore (Arhel et al., 2007). However, more efficient methods are necessary to detect these infrequent complexes within the vast cytoplasm to make this approach practical for addressing biological questions.

Capsid stability affects reverse transcription and infectivity

The viral capsid influences HIV-1 reverse transcription. Biochemical assays of RTCs or PICs from cells do not contain detectable CA, suggesting that the capsid disassembles prior to or during reverse transcription. While these assays may not be able to detect partially intact cores or may strip loosened capsids from the complexes, several CA mutations that altered *in vitro* core stability resulted in reduced reverse transcripts and loss of infectivity in cells, indicating that uncoating and reverse transcription are coupled (Forshey et al., 2002; Rihn et al., 2013; von Schwedler et al., 2003). A CA mutant, N74D, exhibited diminished infectivity in specific cell lines and was hypersusceptible to inhibition by non-nucleoside reverse transcriptase inhibitors (NNRTIs), indicating that core opening may be accelerated for the mutant (Ambrose et al., 2012). Moreover, results from two *in vivo* imaging assays of wild-type HIV-1 using anti-CA antibodies suggested that the majority of the capsid is undetectable within 60 min after virus entry, suggesting that initiation of uncoating precedes the completion of reverse transcription (Hulme et al., 2011; Xu et al., 2013). Collectively, these studies indicate that the proper timing of HIV-1 uncoating is important for productive viral DNA synthesis.

Completion of HIV-1 reverse transcription may require at least partial capsid dissociation. Viral DNA synthesis requires cellular factors (Warren et al., 2012; Warrilow et al., 2009), many of which likely cannot access RTCs prior to capsid opening in the target cell. On the other hand, it has been also hypothesized that reverse transcription itself may promote uncoating, as the size and rigidity of the nascent HIV-1 RNA-DNA hybrid or double-stranded DNA genome may not physically fit inside the core, thus destabilizing it (Hulme et al., 2011). To study the kinetics of HIV-1 uncoating in cells, Hulme and colleagues infected cells expressing TRIMCyp in the presence of cyclosporine A (CsA), which binds to the CypA portion of the restriction factor and prevents it from binding to CA. CsA was subsequently washed out of the cells at different time points in the presence and absence of the NNRTI. The results indicated that delaying reverse transcription prolonged the period of susceptibility to inhibition by TRIMCyp, suggesting that reverse transcription accelerates capsid dissociation. However in other studies, RT inhibitors or mutations in RT that prevent reverse transcription did not alter the kinetics of capsid dissociation *in vitro* and *in vivo* (Kutluay et al., 2013; Xu et al., 2013). Another study suggested that inhibition of reverse transcription reduces the extent of uncoating in target cells and renders HIV-1 resistant to rhTRIM5α (Yang et al., 2013). Although some of these studies appear contradictory, the assays may reflect different detection abilities or different stages of the uncoating process. Indeed, our imaging-based uncoating assays of the CA mutant, E45A, which contains a hyperstable capsid (Forshey et al., 2002; Jun et al., 2011), showed early opening of the core without complete capsid disassembly and accelerated production of reverse transcripts compared to wild-type virus (Xu et al., 2013). Thus, uncoating may occur in multiple steps, with early opening of the HIV-1 capsid preceding its complete dissociation from the viral ribonucleoprotein complex in target cells.

Cellular CypA influences HIV-1 uncoating and nuclear entry

The HIV-1 capsid likely interacts with multiple host cell factors for proper uncoating kinetics and trafficking of the viral genome to the nucleus. The best-characterized human protein that binds to CA cores and facilitates HIV-1 replication is CypA, a peptidylprolyl isomerase that is packaged into HIV-1 virions through interactions with the CA domain of Gag (Luban et al., 1993). CA binds to CypA via an extended NTD loop between helices 4 and 5, with amino acids Gly89 and Pro90 (Gamble et al., 1996) bound at the active site of the enzyme. CypA catalyzes the cis/trans isomerization of the peptide bond between G89 and P90 (Bosco et al., 2002). Although virions incorporate CypA, it is mainly required for HIV-1 infection in the target cell and its packaging into virions does not appear to play a role in replication (Hatziioannou et al., 2005; Sokolskaja et al., 2004). Inhibiting CypA binding to CA by treatment of infected cells with CsA or a similar drug reduces HIV-1 DNA levels, suggesting that CypA may influence uncoating (Braaten et al., 1996b; Ptak et al., 2008). CypA also sensitizes HIV-1 to restriction by rhTRIM5α and PF74, further suggestive of modulation of uncoating.

In vivo selection or mutagenesis studies identified several amino acid substitutions that permit HIV-1 replication in the presence of CsA. These mutations are localized to different areas of the NTD: A92E and G94D in the CypA-binding loop (Aberham et al., 1996; Braaten et al., 1996a), T54A in helix 3, (Yang and Aiken, 2007), N121K between helices 6 and 7 (Takemura et al., 2013), and R132K in helix 7 (Schneidewind et al., 2007). Interestingly, these mutants retain the ability to bind to CypA, and their replication is actually dependent on CsA in some cell lines. For example, CsA is required for replication of these mutants in HeLa and H9 cells but not in Jurkat, HOS, or 293 T cells (Aberham et al., 1996; Braaten et al., 1996a; Hatziioannou et al., 2005; Sokolskaja et al., 2004; Yin et al., 1998). Thus, CypA binding to CA was detrimental on replication in some cells. The infection impairment in HeLa cells is genetically dominant, suggesting that they express a factor(s) that restricts these mutants in a CypA-dependent manner (Song and Aiken, 2007). The CsA-dependent mutant phenotype has recently been linked to another capsid-binding protein, CPSF6 (Henning et al., 2013) (discussed below). However the cell-type dependence of the restriction has not yet been explained.

Lentiviruses, such as HIV-1, exhibit the unusual ability to infect nondividing cells such as macrophages. This phenotype was genetically mapped to the CA protein (Yamashita and Emerman, 2004; Yamashita et al., 2007). Similar to CsA dependence, several cell-cycle dependent CA mutants (A92E, T54A, G94D, R132K) as well as mutants having altered capsid stability (E45A and Q63A/67 A) are selectively impaired for infection of nondividing HeLa cells but not HOS cells or macrophages (Qi et al., 2008; Yamashita and Emerman, 2009). Therefore, CsA dependence requires CypA-CA interaction and differs between cell types. Interestingly, the T54A CA mutant had only partially reduced reverse transcription and showed a defect in nuclear import. An additional mutation in the PF74 binding pocket, A105T, suppresses the CsA-dependent phenotype and restores infectivity, suggesting that allosteric changes distal to the CypA-binding loop alters the CA structure,

thereby changing HIV-1 susceptibility to host restriction (Yang and Aiken, 2007). In addition to restricting HIV-1 at an early stage of the life cycle (e.g., prior to or at reverse transcription), rhTRIM5α can restrict HIV-1 after nuclear entry of the viral DNA (Anderson et al., 2006; Wu et al., 2006). The CypA-CA interaction also contributes to rhTRIM5α restriction in nondividing cells (Yamashita and Emerman, 2009). Thus, CypA also appears to influence nuclear entry and the ability of HIV-1 to infect nondividing cells, which requires translocation of the PIC across an intact nuclear membrane.

HIV-1 entry into nondividing myeloid lineage cells, such as macrophages and dendritic cells (DCs), normally avoids activation of innate immunity triggered by pathogen-associated molecular patterns, such as cytoplasmic double-stranded DNA (Lahaye et al., 2013; Manel et al., 2010; Rasaiyaah et al., 2013). In contrast, infection by the closely related virus HIV-2 induces type 1 interferon (IFN) production in DCs (Lahaye et al., 2013; Manel et al., 2010). The lack of HIV-induced immunity in myeloid cells was shown to be dependent on CA binding to CypA, as pharmacologic inhibition of CypA binding to nascent Gag resulted in an innate immune response (Manel et al., 2010; Rasaiyaah et al., 2013). Similarly, HIV-1 CA mutants with reduced affinity for CypA, such as G89V and P90A, triggered immunity (Manel et al., 2010; Rasaiyaah et al., 2013). In contrast, introduction of a CypA-binding loop into HIV-2 suppressed its IFN activation, akin to HIV-1 (Lahaye et al., 2013). Infection by a subset of HIV-1 CA mutants in both cell types led to upregulation of cytoplasmic DNA sensors that induce IRF3 translocation to the nucleus to induce IFN (Lahaye et al., 2013; Rasaiyaah et al., 2013). These studies demonstrate that HIV-1 escapes innate immune sensing and activation by a stealth mechanism involving CypA-CA interactions. CypA stabilizes the HIV-1 capsid *in vitro* (Shah et al., 2013), suggestive of a capsid-specific mechanism for "cloaking" the viral DNA synthesized during reverse transcription in the cytoplasm.

Activation of innate immune signaling by pathogens results in a strong antiviral response via IFN induction of antiviral genes. One of these encodes the IFN-induced myxovirus resistance 2 (MX2) protein. MX2 inhibits HIV-1 infection by a mechanism involving the viral capsid (Goujon et al., 2013; Kane et al., 2013; Liu et al., 2013). The MX2-mediated block resulted in a reduction in nuclear viral DNA and integration, which also was dependent on HIV-1 CA and CypA. Interestingly, the N74D mutant was less susceptible to inhibition by MX2 than WT HIV-1 (Goujon et al., 2013; Kane et al., 2013), suggesting that CPSF6 may also play a role in MX2 inhibition of infection. Depletion of CypA also prevented MX2-mediated restriction (Liu et al., 2013), suggesting that the CypA binding to the HIV-1 capsid is involved in the antiviral mechanism.

Capsid uncoating influences nuclear import

In recent years, several additional cellular factors have been shown to interact with CA and to affect HIV-1 infection. Transportin 3 (TNPO3) is a karyopherin that binds arginine/serine (RS)-rich proteins and was identified in screens for host factors required for HIV-1 infection (Brass et al., 2008; Konig et al., 2008). The early studies suggested that TNPO3 acts via by binding to IN and promotes trafficking of PICs to the nucleus (Christ et al., 2008). However, the TNPO3 requirement was genetically linked to CA (Krishnan et al., 2010), and infection by CA mutants having altered capsid stability (e.g., E45A, N74D, and the PF74-resistant mutant) was found to be less dependent on TNPO3 (De Iaco and Luban, 2011; Lee et al., 2010; Shah et al., 2013). These studies also showed that TNPO3 depletion results in a defect in integration, rather than nuclear entry. In addition, depletion of cellular TNPO3 reduces the effectiveness of PF74 inhibition of WT HIV-1 but not the TNPO3-independent CA mutants. Furthermore, addition of recombinant TNPO3 to purified HIV-1 cores accelerated their disassembly *in vitro*, and this was further stimulated in the presence of PF74. Interestingly, CypA stabilized HIV-1 cores *in vitro* and antagonized TNPO3-mediated uncoating, suggesting that CypA prevents premature uncoating of capsid in the cytoplasm and later disengages the capsid, permitting completion of uncoating (Shah et al., 2013). Collectively, these observations suggested that TNPO3 and CypA coordinate to control HIV-1 uncoating in target cells, perhaps by directly binding the capsid. However, more recent studies indicate that TNPO3 acts indirectly, by sequestering CPSF6 (see below), and promotes integration rather than nuclear entry (De Iaco and Luban, 2011; De Iaco et al., 2013; Shah et al., 2013).

The cellular protein cleavage and polyadenylation specificity factor 6 (CPSF6) is part of an mRNA processing complex that shuttles between nucleus and cytoplasm (Ruegsegger et al., 1998). Truncating the carboxyl-terminal region of CPSF6 (CPSF6-358) results in a protein that is largely cytosolic and inhibits HIV-1 infection by preventing nuclear entry (Lee et al., 2010). Selection for resistance to CPSF6-358 resulted in emergence of a virus containing the N74D CA substitution, which inhibits binding to CPSF6-358 (Lee et al., 2010; Price et al., 2012). Interestingly, the CPSF6-binding pocket in CA overlaps the binding site for PF74 (Blair et al., 2010; Price et al., 2012), suggesting that the compound may compete for CPSF6 binding in target cells.

HIV-1 uses the nuclear pore to gain entry into the nucleus by a mechanism that depends on the viral capsid. The nuclear pore complex includes nuclear pore proteins NUP153 and NUP358 (or RanBP2), both of which were shown to be required for HIV-1 infection in the same screens that identified TNPO3 (Brass et al., 2008; Konig et al., 2008). However, infection by the CPSF6-independent mutant N74D HIV-1 does not depend on these nucleoporins (Lee et al., 2010). Recently, it was shown that CA binds directly to the NUP358 cyclophilin domain (Schaller et al., 2011) and to NUP153 (Matreyek et al., 2013). Genetic studies indicate that NUP153 interacts with a region of CA that overlaps with the site at which PF74 and BI-2 bind (Matreyek et al., 2013). It is possible that cytoplasmic pools of capsid-binding NUPs may control uncoating by engaging the capsid. Alternatively, NUPs may act directly on the core after docking to the nuclear pore. These hypotheses are not mutually exclusive.

Price et al. proposed that CPSF6 binding to the HIV-1 capsid facilitates its use of nuclear import factors such as TNPO3 and nucleoporins (Price et al., 2012). Several lines of evidence support this hypothesis. Two groups independently showed in cellular and biochemical assays that TNPO3-dependence of HIV-1 infection is correlated with the ability of CPSF6 to bind CA (De Iaco et al., 2013; Fricke et al., 2013). Like N74D HIV-1, the A105T mutant is located in the CPSF6/PF74-binding pocket and renders HIV-1 resistant to inhibition by CPSF6-358 (De Iaco et al., 2013). Moreover, both mutations restore infectivity to the cell-cycle dependent T54A mutant (Henning et al., 2013). Collectively, studies of CPSF6-358-resistant mutations (N74D or A105T) and CypA-independent or CsA-dependent mutations (G89V and G94D) suggest that CPSF6 and CypA bind independently to HIV-1 capsid (Ambrose et al., 2012; Henning et al., 2013), though the current evidence for this is indirect.

A recent study showed that endogenous CPSF6 can inhibit multiple CA mutants, including a naturally occurring double mutant, R132K/L136M (Henning et al., 2013), which is selected in patients who have the human leukocyte antigen (HLA)-B27 allele (Henning et al., 2013; Schneidewind et al., 2007). The HLA-B27 allele encodes a particular major histocompatibility complex (MHC) class I protein that presents certain peptides to CD8+ T cells, and is associated with protection against AIDS progression (Gao et al., 2005; Hendel et al., 1999). However, two CA mutations, R132K and L136M arise in during infection of B27+ individuals

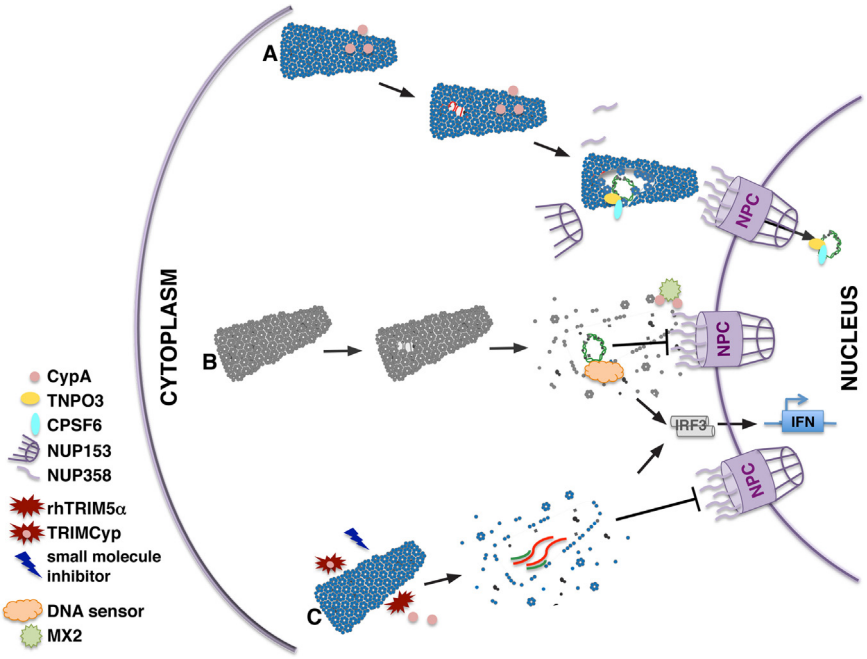

Fig. 1. HIV-1 uncoating is linked to reverse transcription and nuclear import and requires host cell factors. (A) Core opening likely involves multiple steps and utilizes CypA in a cell type-dependent manner to protect the viral DNA genome until nuclear entry, which is facilitated by CA binding of host cell proteins TNPO3, CPSF6, and components of the nuclear pore complex (NUP153 and NUP358). (B) CA mutants, CypA depletion, and CPSF6 depletion induces premature uncoating distal from the nucleus. Exposed viral DNA stimulates an innate immune response via detection of DNA sensors. The resulting IFN response induces expression of MX2, a host factor that prevents viral DNA nuclear entry. (C) Uncoating is accelerated by small molecule inhibitors and nonhuman primate restriction factors, rhTRIM5α and TRIMCyp. Premature uncoating leads to reduced reverse transcription and little nuclear import of viral DNA.

and result in HIV-1 escape from cytotoxic T lymphocytes. These substitutions were associated with accelerated disease progression (Kelleher et al., 2001). Like N74D, the R132K/L136M variant requires neither TNPO3 for infection nor either NUP153 or NUP358 for nuclear entry. *In vivo*, compensatory mutations in CA other than N74D emerge that conserve the dependence on these host proteins, which suggests that CPSF6 and TNPO3/nucleoporin use is advantageous for HIV-1 persistence in the host (Henning et al., 2013).

Models of uncoating

Although there are many unanswered questions regarding HIV-1 uncoating, the collective data suggest a model (Fig. 1). First, based on the recent studies of HIV-1 DNA sensing in myeloid cells (Lahaye et al., 2013; Rasaiyaah et al., 2013), the model posits that CypA prevents premature uncoating, thus facilitating reverse transcription and subsequent engagement of the host proteins TNPO3 and CPSF6 and subsequently NUP358 and NUP153, thereby permitting uncoating and transport of PICs through nuclear pores (Fig. 1A). NUPs, including NUP358 containing a CypA motif (Schaller et al., 2011), are required for proper uncoating and nuclear trafficking. These results suggest that uncoating occurs at or near the nuclear pore to allow safe passage through the cytoplasm without detection by DNA sensors (Rasaiyaah et al., 2013). This view is supported by a previous study of HIV-1 infection of cells using confocal and electron microscopy techniques (Arhel et al., 2007).

Exposure of cells to some CA mutants induces type 1 IFN production (Manel et al., 2010; Rasaiyaah et al., 2013) apparently by undergoing premature uncoating (Fig. 1B). For example, the N74D and P90A CA mutants fail to engage CPSF6 and CypA, respectively, possibly resulting in rapid uncoating and exposure of viral DNA, thus allowing detection by DNA sensors, such as cGAS, and activation of innate immune responses. HIV-1 infection of CPSF6- or CypA-depleted macrophages also induces type 1 IFN secretion, which can induce MX2-mediated inhibition of nuclear entry and integration.

Finally, premature uncoating by inhibitors and simian restriction factors, such as rhTRIM5α and TRIMCyp, aborts reverse transcription (Fig. 1C). It is possible that either exposed viral RNA-DNA hybrids or small amounts of single- or double-strand viral DNA could stimulate innate immunity. A lack of functional PICs associated with CA, CPSF6, and TNPO3 will result in little integrated viral DNA.

Conclusions and remaining questions

HIV-1 uncoating appears to be a tightly controlled process that depends on proper capsid stability and interaction with multiple host factors. Premature or delayed dissociation of capsid reduces production of viral DNA and nuclear import of viral DNA, resulting in abortive infection. Delayed uncoating also appears to be deleterious to infection. Uncoating is a promising target for antiretroviral therapy by small molecule inhibitors, and perturbing this process may also allow virus control by IFN-induced restriction factors.

Rapid progress on HIV-1 capsid structure and its interactions with host factors has been made in the past decade. Nevertheless, much remains to be learned. For example, molecular and spatiotemporal aspects of uncoating, and the role of host factors in this process, remain poorly understood. The mechanism by which CypA protects the viral genome on its journey to the nucleus and enhances inhibition by restriction factors is unclear. Studies of uncoating in target cells are complicated by the apparently high percentage of nonfunctional particles and by the small number of cores that enter cells. New imaging technologies are needed to define the precise steps of uncoating and where they occur. Further research should also focus on the mechanism by which

the capsid binds and utilizes positive-acting host factors and how it traverses the nuclear pore. Finally, differences in expression of host cofactors for HIV-1 uncoating and nuclear import are unknown, and it remains to be seen if interventions will be effective in both $CD4^+$ *T* cells as well as macrophages. Further studies of HIV-1 capsid structure, stability, and interaction with host factors will certainly lead to fascinating new insights about this formidable human pathogen.

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