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Nuclear Capsid Uncoating and Reverse Transcription of HIV-1

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Abstract

After cell entry, human immunodeficiency virus type 1 (HIV-1) replication involves reverse transcription of the RNA genome, nuclear import of the subviral complex without nuclear envelope breakdown, and integration of the viral complementary DNA into the host genome. Here, we discuss recent evidence indicating that completion of reverse transcription and viral genome uncoating occur in the nucleus rather than in the cytoplasm, as previously thought, and suggest a testable model for nuclear import and uncoating. Multiple recent studies indicated that the cone-shaped capsid, which encases the genome and replication proteins, not only serves as a reaction container for reverse transcription and as a shield from innate immune sensors but also may constitute the elusive HIV-1 nuclear import factor. Rupture of the capsid may be triggered in the nucleus by completion of reverse transcription, by yet-unknown nuclear factors, or by physical damage, and it appears to occur in close temporal and spatial association with the integration process.

1. INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is an enveloped retrovirus containing two plus-strand RNA molecules inside the cone-shaped mature capsid together with the viral replication enzymes. Once the virus enters a cell by fusion with the cell membrane, genome replication commences inside the capsid by reverse transcription of the RNA genome into a double-stranded DNA (dsDNA), also termed complementary or copy DNA (cDNA), which eventually integrates into host chromatin, leading to permanent infection of the cell and its daughter cells. Reverse transcription is catalyzed by the virally encoded reverse transcriptase (RT) as part of the reverse transcription complex (RTC). Integration of the viral cDNA is mediated by the viral integrase (IN) as part of the preintegration complex (PIC). HIV-1—in contrast to some other retroviruses—productively infects nondividing cells. Thus, a subviral complex comprising the genome must access the nucleus without nuclear envelope breakdown, but the composition of this complex is not yet fully defined. For integration to occur, the capsid needs to be shed in a process termed uncoating, but the timing and mechanism of uncoating and the role of the capsid in early replication have been debated (1–6). Until very recently, completion of reverse transcription and uncoating were believed to occur in the cytosol. In this review, we focus on recent evidence indicating nuclear uncoating and completion of cDNA synthesis. For details on the mechanism of reverse transcription, we refer the reader to other comprehensive reviews (7, 8).

The mature HIV-1 capsid is composed of ~250 hexamers and 12 pentamers of the viral capsid protein (CA), arranged into a fullerene cone (9, 10) (**Figure 1*a***). Seven pentamers at the wide end and five at the narrow end induce curvature and enable closure of the structure (11, 12) (**Figure 1*a***). The capsid contains at least three conserved interfaces that mediate interaction with host cell factors (**Figure 1*b–e***): (*i*) a hydrophobic binding cleft encompassing neighboring CA subunits within a hexamer (**Figure 1*b*,*d*,*e***), (*ii*) the R18 pore at the center of the hexamer (**Figure 1*b*,*c***), and (*iii*) a cyclophilin A (CypA)–binding loop on each CA subunit (**Figure 1*b*,*d***). Furthermore, the restriction factor tripartite motif 5α (TRIM5α) can form a large secondary hexagonal lattice on the capsid. Binding of TRIM5α with its SPRY domain to the capsid lattice spanning six CA hexamers leads to proteasomal degradation (13–15). CypA binding to the capsid can prevent TRIM5α restriction (16).

The past 30 years have seen a constant evolution of our perspective regarding HIV-1 capsid integrity and uncoating. This started in the 1990s with pioneering experiments by Haseltine and Fassati, who biochemically characterized HIV-1 RTC/PIC isolated from infected cells (17–20) (Section 2), and proceeded with labeling of subviral complexes by Hope and colleagues in the early 2000s (21–23) (Section 3) to recent studies applying advanced microscopic methods (Section 4). In this review, we attempt to reconcile the data of earlier reports with today's insights and propose models regarding nuclear import, completion of reverse transcription, and uncoating of the viral genome.

2. HISTORICAL PERSPECTIVE ON CYTOSOLIC COMPONENTS OF THE REVERSE TRANSCRIPTION COMPLEX/PREINTEGRATION COMPLEX AND SEARCH FOR THE NUCLEAR IMPORT FACTOR

Initial attempts to define the composition of HIV-1 RTC/PIC focused on biochemically isolated subviral complexes (17–20). Viral cDNA extracted from the cytosol of newly infected cells was found to be associated with IN, and these complexes were competent for integration in vitro (18). Neither CA nor RT was detected in the complexes by immunoblotting (18), but association with RT activity indicated the presence of this enzyme (19). The observation that functional RTC, biochemically isolated from the cytosol of infected cells, lacked CA suggested that uncoating occurs

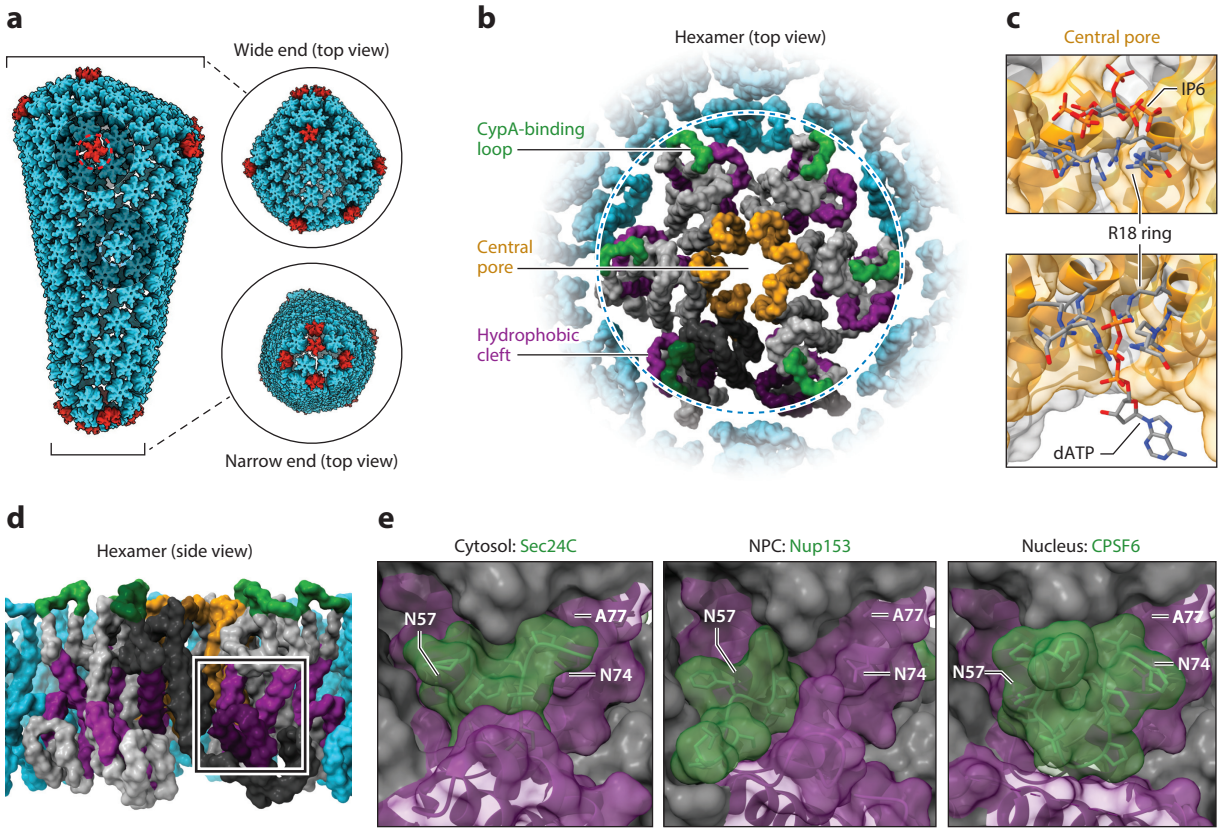

Figure 1

Structure and interaction interfaces of the HIV-1 capsid. (*a*) Complete model of the mature HIV-1 capsid, encompassing ~250 CA hexamers (*blue*) and 12 CA pentamers (*red*). The model was built from cryo-electron microscopy density maps of hexameric and pentameric units: EMD-3465 (12). HIV-1 capsid cone model courtesy of Simone Mattei. (*b*) Top view of the hexameric CA assembly (PDB: 5mcx) (12) highlighting conserved interaction interfaces. Orange indicates the central R18 pore (CA residues 5–29); purple indicates the hydrophobic cleft (residues 50–80 of one CA subunit and residues 160–190 of a neighboring CA subunit); and green indicates the CypA-binding loop (residues 88–96). Surrounding hexamers are colored in blue, and a single CA monomer is visualized in a darker shade. (*c*) The central R18 pore in complex with IP6 (*top panel*; PDB: 6bhs) (150) and dATP (*bottom panel*; PDB: 5hgm) (75). The transparent surface and ribbon representation of residues 5–29 are colored in yellow. The positively charged R18 residues, IP6 and dATP are rendered in stick representation (C atoms, *gray*; O, *red*; P, *orange*; N, *blue*). (*d*) Side view of the hexameric CA assembly shown in panel *b*. The white box indicates the part of the hydrophobic binding cleft shown in panel *e*. (*e*) Structures of CA binding regions of Sec24C (*left*, *green*; PDB: 6pu1) (113), Nup153 (*middle*, *green*; PDB: 4u0c) (107), and CPSF6 (*right*, *green*; PDB: 4u0a) (107) in complex with the CA hexamer. The hydrophobic binding cleft is colored in purple and rendered in transparent surface representation with underlying ribbon visualization of the secondary structure. CA residues N57, N74, and A77, directly involved in host factor binding (66, 81, 95, 103), are rendered in stick representation. Abbreviations: CA, capsid protein; CPSF6, cleavage and polyadenylation specificity factor 6; CypA, cyclophilin A, dATP, deoxyadenosine triphosphate; HIV-1, human immunodeficiency virus type 1; IP6, inositol hexakisphosphate; NPC, nuclear pore complex; Nup153, nucleoporin 153; PDB, Protein Data Bank.

early after virus entry and prior to reverse transcription; this was supported by the observation that HIV-1 capsids immediately disintegrate upon detergent stripping of the lipid membrane of complete virions (24–26). On the contrary, studies demonstrating that certain point mutations in CA affected reverse transcription, and—vice versa—that blocking reverse transcription impaired uncoating, argued for a temporal and functional connection between these processes (27–33). These

findings supported a model of delayed uncoating, either gradually during cytosolic trafficking of subviral complexes or directly before nuclear entry. A pioneering study employed correlative light and electron microscopy (CLEM) to visualize actively reverse transcribing complexes from the cytosol of infected cells. It revealed large complexes trafficking along microtubules, but their exact structure and composition could not be determined (21). Transport of the capsid through intact nuclear pores was not considered at the time because its size exceeded the assumed limit of the nuclear pore complex (NPC) channel.

A key issue addressed in many studies was the nature of the nuclear import mechanism for the HIV-1 RTC/PIC. These analyses mostly relied on quantitation of 2-long terminal repeat (LTR) circles, a dead-end product that results from circularization of full-length HIV-1 cDNA via the non-homologous end joining pathway in the nucleus (34), as surrogate readout of nuclear import. Canonical nuclear import requires a karyophilic, or nuclear localization, signal (NLS) (35). Potential NLSs were identified within the HIV-1 MA (matrix) protein (36–38) and the bona fide RTC/PIC components IN (39) and Vpr (40, 41). Because some of these proteins display predominantly nuclear localization when expressed alone and outside of the viral context (42), they were discussed as potential nuclear import factors of the RTC/PIC. Other studies suggested a critical role for the DNA flap, a structural intermediate of reverse transcription, in HIV-1 nuclear import (43, 44). However, further studies did not support an essential role for either of these hypothetical import factors (45–50). A comprehensive analysis found neither of these components alone, nor a combination thereof, to be required for HIV-1 nuclear import; only deletion of the flap resulted in some delay in 2-LTR circle formation (51). The search for a nuclear import factor of the HIV-1 RTC/PIC was further expanded to host cell proteins, and it was suggested that the canonical import factor Importin 7 (52) as well as transfer RNA (tRNA) (53) and Transportin 3 (54) played a role. The contribution of Transportin 3 to nuclear entry of HIV-1 subviral complexes was subsequently disputed as well (55). In summary, these studies did not yield a conclusive model for the nuclear import of HIV-1 replication complexes and did not identify an essential import signal or import factor.

3. THE QUEST FOR THE SITE OF CAPSID UNCOATING

More sophisticated uncoating assays later indicated that the viral capsid may be retained, at least for some time, on the cytoplasmic subviral particle and suggested a role of capsids in early replication. The fate of capsid assay (56, 57) involved ultracentrifugation of post-entry cell extracts through a dense sucrose layer to separate pelletable CA associated with viral nucleoprotein complexes from free CA, and suggested the presence of CA-associated subviral complexes inside the cytosol. The cyclosporine A (CsA) washout assay employed cells expressing the chimeric restriction factor Trim-Cyp (58), which specifically binds the HIV-1 capsid through the CA Cyp-binding loop and targets it for proteasomal degradation. Controlled induction of Trim-Cyp restriction by washout of the competing compound CsA revealed a time-dependent loss of sensitivity against capsid-targeted restriction, indicating gradual uncoating in the cytosol (57, 59).

While these results suggested the presence of capsid-encased subviral complexes in the cytosol of infected cells, most early electron microscopy (EM) studies, including our own, readily detected complete virions inside endosomes but failed to detect cone-shaped capsids in the cytosol. However, visualization of rare structures in the vast electron-dense cytosolic environment is very difficult to achieve. The first study applying CLEM to identify subviral structures by EM (21) relied on extraction of cytoskeletal structures and scanning EM (SEM), substantially limiting spatial and structural resolution; this study did not provide evidence for cytosolic capsids. Another SEM study detected capsid-shaped objects associated with the nuclear envelope (60) but employed

an extraction procedure that could have led to deposition of plasma membrane–associated and endosomal material on the nuclear envelope. Subsequent technological advances enabled the application of CLEM analysis in infected cells (61). One study (61) reported a small number of capsid-shaped objects in the cytosol, but only for a variant carrying a capsid-stabilizing mutation in CA, further illustrating the difficulty of visualizing the capsid on entering subviral structures. The large proportion of nonproductive (endosomal) cell-associated virus particles that need to be distinguished from cytosolic post-fusion intermediates further confounds such analyses.

Mamede et al. (27) developed a fluorescence microscopy–based assay to correlate the integrity of the incoming capsid with subsequent productive infection of the respective cell. To this end, green fluorescent protein (GFP) was incorporated into the virion as an internal fluid-phase marker, and cells were infected at a low multiplicity of infection to ensure that individual cells underwent only a single entry event that could be correlated with subsequent productive infection. These authors observed a two-step loss of the GFP marker, which was interpreted as (*a*) fusion and loss of CA not incorporated into the capsid and (*b*) subsequent uncoating of the capsid lattice with loss of internal GFP; only cells undergoing this two-phase loss of GFP were later found to be productively infected (27). However, Li et al. (62) revisited this question using the same approach but reported that capsid integrity remained largely intact in the cytosol.

Early capsid disintegration in the cytosol cannot easily be reconciled with the findings that (*a*) CA is critical for HIV-1 for infection of nondividing cells (63, 64), (*b*) some CA mutants display defects in post-entry steps (65–67), and (*c*) CA-host factor binding affects early viral replication (68, 69). Rather, these observations indicate that at least part of CA is retained during cytoplasmic trafficking, potentially until nuclear import. This conclusion was supported by the discovery that the capsid can protect the viral genome from innate immune detection by cytosolic DNA sensors (70–72). Even partial disintegration of the capsid, as indicated by loss of the fluid phase GFP marker, should allow cytosolic DNA sensors access to the viral nucleic acid. However, this was not observed unless the capsid was pharmacologically permeabilized (72).

As discussed above, the observation that isolated HIV-1 particles in vitro immediately lose their capsid upon stripping the viral membrane strongly suggested rapid cytosolic uncoating. Strikingly, however, isolated HIV-1 capsids were later found to be stable in the presence of the metabolite inositol hexakisphosphate (IP6), transforming their half-life from minutes to many hours (73, 74). IP6 binds to a prominent pore in the center of the CA hexamer lined by six positively charged arginine (R18) residues contacting each other (**Figure 1*c***). It has been suggested that this pore allows import of deoxynucleoside triphosphates (dNTPs) into the capsid, thereby promoting reverse transcription (75, 76) (**Figure 1*c***). Molecular dynamics simulations indicated that cooperative binding of multiple dNTPs to the central pore may translocate them into the core (77). Interestingly, Huang et al. (78) identified a host protein, FEZ1, that binds to the R18 pore via negatively charged glutamate residues potentially competing with dNTPs and IP6. FEZ1 is a kinesin adapter and can mediate capsid transport on microtubules (79). It is tempting to speculate that FEZ1, or another R18 pore binding protein, might regulate the degree of reverse transcription (Section 6).

The high sensitivity of HIV-1 CA to mutations (80) impairs functional fluorescent CA labeling. Most analyses of CA content of subviral particles therefore relied on antibody detection by immunofluorescence. Ideally, these studies would allow distinction of cytosolic from (nonproductive) endosomal structures and should identify subviral complexes undergoing productive reverse transcription. Many recent studies showed a clear colocalization of strong CA signals with cytosolic subviral particles identified by fluorescent replication proteins (33, 60, 70, 81–88). Metabolically labeling the newly synthesized cDNA indicated that actively reverse transcribing post-fusion cores also exhibit a strong CA signal with a similar intensity as virion-associated bona fide capsids

(81, 82). Direct support for retention of the capsid on cytoplasmic subviral HIV-1 particles came from a recent CLEM study in the T cell line SupT1-R5. Cytoplasmic structures identified by a fluorescent IN fusion clearly revealed the typical cone-shaped capsid of HIV-1 with the electron-dense viral nucleoprotein complex inside (89). This was observed independent of cytosolic localization, including in direct vicinity to nuclear pores. Taken together, these data provided strong evidence that most of the CA content, as well as the shape of the capsid shell, is retained on cytosolic subviral HIV-1 complexes, indicating that the ill-defined cytoplasmic RTC is in fact the intact viral capsid, at least until reaching the nuclear pore.

In conclusion, the HIV-1 capsid is a metastable structure stabilized by the high IP6 concentration (10–100 μM) in the cytosol (73). The capsid thus appears to provide the enclosed space supporting reverse transcription by concentrating dNTPs, preventing loss of replication factors, and facilitating strand transfer and recombination events while protecting the genome from degradation and immune sensing. Providing the interface for interaction with host cell factors, the capsid also mediates cytosolic transport processes along microtubules (90, 91).

The results discussed so far argue against immediate or delayed uncoating of subviral cytosolic complexes. Given that the width of the capsid (~60 nm at the broad end) is larger than the reported diameter of the central channel of isolated NPCs [~43 nm (92)], the most likely location for uncoating would be directly at the nuclear pore. Uncoating of the capsid at the nuclear pore was indeed reported for other viruses with nuclear genome replication, including adenovirus and herpes simplex virus (93). Recent reports suggested that HIV-1 subviral particles also lose their CA content upon nuclear pore entry. This conclusion was mainly based on indirect detection of the capsid by a fluorescent fusion of the CA-binding host protein CypA. These experiments revealed strong labeling of cytosolic subviral complexes by the CypA fusion with loss of the fluorescent signal at the nuclear pore, suggesting uncoating at this site (86, 94). Given that CA detection was indirect, however, an alternative interpretation would be CypA displacement from CA, especially considering that the CA-binding nucleoporin (Nup) Nup358 also contains a Cyp domain, which may compete for binding to the Cyp-binding loop in CA (see Section 5).

Immunofluorescence studies from many laboratories reported the complete or largely complete absence of CA from subviral HIV-1 complexes inside the nucleus in most cell types (29, 82, 83), suggesting uncoating to be associated with nuclear entry. Some cell type–dependent differences were reported regarding the presence of nuclear CA. No or very weak CA signals were detected on nuclear complexes in T cell lines (83), while HeLa-based cells showed low but varying amounts of nuclear CA (29, 82, 95). This was different in monocyte-derived macrophages (MDMs), where we (81–83) and others (96–98) detected strong nuclear CA signals approaching the signal intensity of the bona fide HIV-1 capsid stained in parallel. These results argued for cell type–dependent differences in nuclear import and uncoating and suggested that capsid-derived structures retaining much of the CA content may enter the nucleus without nuclear envelope breakdown, at least in MDM.

4. RECENT EVIDENCE FOR NUCLEAR ENTRY OF CAPSID-LIKE STRUCTURES

4.1. Nuclear HIV-1 Subviral Complexes Largely Retain the CA Content of the Incoming Capsid

Recent studies from several groups employing immunofluorescence with different extraction protocols (81, 95–97, 99), incorporation of labeled CA into the capsid (62, 85, 100), and CLEM of subviral complexes at different subcellular localizations (89, 99, 100) suggested that capsid-encased complexes can indeed enter the nucleus without prior uncoating in all cell types analyzed. The

strongest evidence against nuclear capsids or capsid-derived structures with high CA content came from (*a*) immunofluorescence studies described in the previous section and (*b*) the dimensions of the HIV-1 capsid exceeding the reported size limit of the nuclear pore. New evidence challenged these prior conclusions and is discussed in this and the following section.

Lack of antibody detection of nuclear CA may be caused not only by disassembly of the capsid but also by loss of or shielding of antigenic epitopes. Interestingly, studies detecting a strong CA signal on nuclear HIV-1 complexes generally applied harsh extraction conditions or partial digestion prior to antibody detection. Chin et al. (95) subjected their samples to partial protease digestion prior to antibody detection and observed CA on nuclear complexes. Furthermore, the click reaction for detection of 5-ethynyl-2′-deoxyuridine (EdU)-labeled HIV-1 DNA requires copper catalysis, which strongly extracts cells (101). This technique was mostly applied in nondividing MDM and also yielded clear nuclear CA signals (81–83, 96–98, 102). Thus, weak and apparently cell type–dependent nuclear CA signals could be caused by inaccessibility of antigenic epitopes during standard immunofluorescence analyses rather than by loss of the capsid shell.

This conclusion was supported by experiments addressing the nuclear host protein cleavage and polyadenylation specificity factor 6 (CPSF6), which specifically binds the hexameric CA lattice (99). Nuclear HIV-1 complexes were found to be strongly decorated with CPSF6 (81, 83, 95, 103, 104), and cytoplasmic expression of truncated or mutated CPSF6 blocked HIV-1 prior to nuclear import of the replication complex (105). Given the specificity of CPSF6 for the assembled capsid lattice, these results indicated CA-dependent recruitment of CPSF6 to an—at least partially—assembled CA lattice on nuclear subviral complexes. CPSF6 binding to the HIV-1 subviral complex can be abolished by mutations in CA targeting the CPSF6 binding site (81, 83, 95, 103, 104) (**Figure 1*e***) or by the small molecule inhibitor PF74 (99), competitively binding the same hydrophobic pocket (106–108) and displacing CPSF6 (99). Treatment of infected cells with PF74 after nuclear entry of the subviral complex reconstituted immunodetection of CA with signals similar to bona fide capsids, directly showing that lack of immunodetection of CA on nuclear HIV-1 complexes was due to epitope shielding by CPSF6 (99). Accordingly, Dharan et al. (109) employed a system to transiently block passage of the subviral complex through nuclear pores and observed that PF74 retained anti-HIV-1 activity even after reverting the import block. Furthermore, biochemical analysis of fractionated cell extracts corroborated the existence of an at least partially intact CA lattice inside the nucleus (110).

Similar conclusions were derived from studies with directly labeled CA variants. Burdick et al. (85) used a GFP-labeled CA variant that gives rise to infectious HIV-1 when complemented with an excess of wild-type CA. These authors observed fluorescent subviral particles approaching the nuclear envelope and entering the nucleus, with clear detection of nuclear signals for the GFP-CA fusion until shortly before integration (85). Importantly, they also provided direct evidence that these nuclear complexes gave rise to productive infection of the respective cell and thus constituted functional HIV-1 replication complexes. Engineering an HIV-1 variant carrying a click-labeled non-natural amino acid in CA by genetic code expansion allowed a more quantitative analysis of the CA content of HIV-1 complexes at different subcellular localizations (100). Strong CA signals similar to those of bona fide capsids were detected in the nucleus of HeLa-based and T cell lines as well as in primary $CD4^+$ T cells, even at conditions where CA was undetectable by antibody staining (100). Thus, CA remains associated with nuclear HIV-1 subviral complexes in all cell types analyzed, and the reported variable results were probably due to epitope shielding by host proteins. While the described results clearly indicated that CA is largely retained on nuclear HIV-1 complexes, they do not provide information on whether this represents a regular HIV-1 capsid lattice, a remodeled capsid-like structure, or remaining CA hexamers (stabilized by, for example, CPSF6 binding). Addressing this question requires ultrastructural analysis of the respective complexes.

4.2. Nuclear Pores Are Sufficiently Large to Accommodate Morphologically Intact HIV-1 Capsids

Recent CLEM and electron tomography (ET) analysis of high-pressure frozen and freeze-substituted Sup-T1 T cells infected with HIV-1 revealed cone-shaped capsids comprising electron-dense nucleoprotein adjacent to the nuclear envelope and partially entering nuclear pores with their narrow ends first (89) (**Figure 2**). Morphologically intact capsids were also observed deep inside intact nuclear pores, with the octameric NPC assembly surrounding the viral capsid in the central channel (89). The presence of apparently intact capsids inside nuclear pores was confirmed by focused ion beam (FIB) milling and cryo-electron tomography (cryo-ET) of native flash-frozen cells (89). Sub-tomogram averaging revealed that the regular capsid lattice was at least partially retained. A full lattice could not be detected, but the dense cellular environment did not allow achievement of the same high resolution as for the isolated mature virion.

Capsid-related structures were also detected inside the nucleus of infected cells using CLEM and ET (89, 99, 100). They sometimes closely resembled intact conical capsids with electron-dense nucleoprotein inside, while others appeared broken and often tubular and had lost the

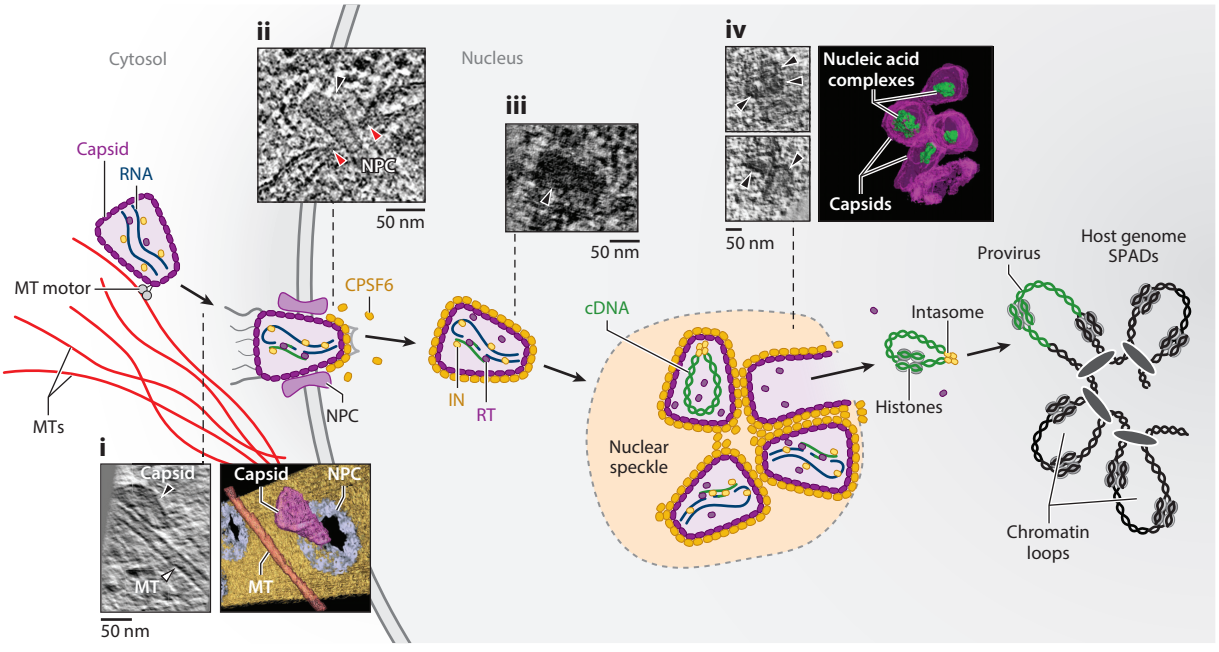

Figure 2

Post-entry steps of HIV-1 early replication. After cytosolic entry, reverse transcription of the HIV-1 RNA genome into cDNA is initiated within the protected environment of the capsid shell. The capsid utilizes MTs and associated motors to reach and dock to NPCs. Subsequent interactions of the CA lattice with NPC components and the nuclear host factor CPSF6 mediate translocation of the capsid through the NPC, retaining its cone-shaped morphology. CPSF6 then mediates accumulation of capsids in nuclear speckles, where plus-strand synthesis of the viral double-stranded cDNA is likely completed. Physical disruption of the capsid releases the completed cDNA for integration into the host cell genome, located in the vicinity of the uncoating site. Remnants of the broken capsid are not part of the cDNA complex and remain as distinct structures in the nucleus for prolonged times after uncoating. Black, white, and red arrowheads indicate the capsid, MT cross section, and NPC, respectively. Abbreviations: CA, capsid protein; cDNA, complementary DNA; CPSF6, cleavage and polyadenylation specificity factor 6; HIV-1, human immunodeficiency virus type 1; IN, integrase; MT, microtubule; NPC, nuclear pore complex; RT, reverse transcriptase; SPAD, speckle-associated domain. Electron micrographs i and ii adapted from Reference 89 (CC BY-NC-ND 4.0), and electron micrograph iv adapted from Reference 99 (CC BY 4.0).

electron-dense structure inside. Both apparently intact and broken empty structures were also detected upon CLEM and ET of cells infected with the click-labeled CA variant, obtained by genetic code expansion (100). We interpret the broken structures as ruptured capsid remnants after nuclear uncoating, as discussed in Section 7.

Strong evidence for nuclear import of CA-associated subviral complexes came from live-cell imaging of HIV-1 containing a GFP-CA fusion protein, which showed nuclear entry of the GFP signal without cell division (85). This is consistent with experiments in HeLa-based cells carrying fluorescent lamin that were blocked for cell division. Analysis of the history of cells displaying nuclear CA complexes confirmed that these cells had not undergone nuclear envelope breakdown; thus, nuclear subviral complexes had entered through intact nuclear pores (99).

How do these morphologically intact-appearing capsid-like structures gain access to the nucleus given the described size restrictions? This question was solved by cryo-ET of FIB-milled HIV-1 infected and uninfected T cell lines and primary $CD4^+$ T cells. Compared to isolated nuclear pores, the central channel of nuclear pores in situ was found to be wider with an inner ring diameter of ~64 nm (89). This is consistent with a recent report describing a flexible width of the central channel of yeast nuclear pores. This report suggested that the NPC scaffold is mechanosensitive and its diameter is regulated by membrane tension (111). The wider diameter and flexibility of the NPC were additionally confirmed in human cells (112). Isolated nuclear pores may thus represent the collapsed status, while the pores in intact T cells represent a dilated state due to the nuclear envelope tension. No significant difference was observed in average pore diameter between infected and uninfected T cells (89), arguing that the dilated state is not induced by the virus. The observation of dilated nuclear pores in intact T cells showed that the complete capsid with a maximum diameter of ~60 nm can be accommodated in the central channel and thus explain the presence of morphologically intact capsids and capsid-like structures in the nucleus of nondividing cells. This leaves several questions that we address in the following sections: What is the molecular mechanism of capsid nuclear import? What is the temporal and functional relationship between reverse transcription, nuclear import, and capsid uncoating? And what are the nature, molecular mechanism, and site of the uncoating event?

5. A MODEL FOR NUCLEAR IMPORT OF THE HIV-1 CAPSID

The evolution of the defined and elaborate capsid structure that needs to assemble within the confined and crowded environment of the mature virion suggests that the conical shape may convey some selective advantage. We propose that transport of the HIV-1 capsid toward and through the nuclear pore is governed by a handover model, which provides a theoretical rationale for the distinct capsid architecture (**Figure 3**). This model assumes sequential binding of several host factors to the capsid lattice promoting its nuclear import. The same hydrophobic binding cleft within the capsid lattice (**Figure 1*b*,*d*,*e***) has been shown to interact with cytosolic, nuclear, and NPC proteins (106–108, 113). Consecutive and competitive binding of these host proteins may thus direct the capsid to the nucleus; displacement may occur due to higher affinity and/or higher local concentration of the competing factor. The narrow end of the cone may facilitate threading the capsid into the central channel, while the exposure of additional Nup binding sites on the capsid lattice may drive translocation toward the nucleus when the increasingly wider parts of the cone enter into the channel.

In the cytosol, the incoming capsid encounters two host proteins that recognize distinct structural features. The coat protein complex II component Sec24C was recently shown to bind the hydrophobic cleft via a phenylalanine-glycine (FG) motif (**Figure 1*e***), enhancing HIV-1 capsid stability; its depletion affects post-entry stages of the virus (113). FG motifs mediate weak

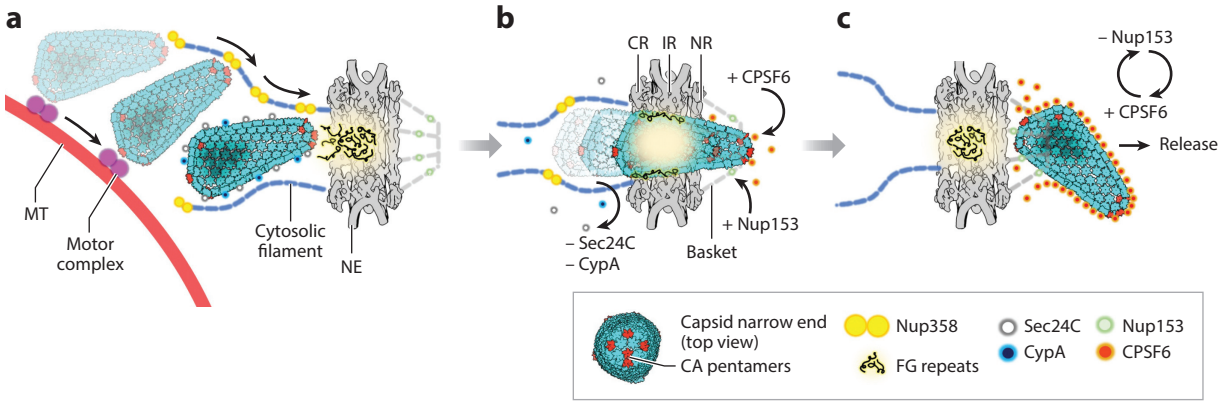

Figure 3

Proposed model of HIV-1 nuclear import. (*a*) Docking. Having arrived at the NPC, MT-associated capsids are directed toward the central channel via interaction with Nup358, the major component of cytosolic NPC filaments. Nup358 interacts with the capsid lattice and recognizes CA pentamers to move and orient the narrow end toward the NPC and into the dense hydrophobic meshwork of FG-Nups in the central channel. (*b*) NPC channel penetration. During this process cytosolic host factors (including CypA and Sec24C stabilizing the capsid) may be displaced from the capsid by Nup358 and FG repeats, respectively. Sequential binding of FG repeats immerses the capsid in the unstructured hydrogel-like liquid phase within the NPC channel. Subsequently, the narrow end is exposed to the nuclear basket region, where it interacts with Nup153 followed by CPSF6. (*c*) Translocation to the nucleoplasm. The final step of capsid nuclear import is mediated by interaction of the hexameric lattice with Nup153 and CPSF6, pulling the capsid out of the channel and blocking retrograde movement. Finally, a (potentially stabilizing) CPSF6 coat decorates the capsid surface, releasing the capsid into the nucleoplasm and directing it to the site of integration. The scheme was created using cryo-EM density maps of hexameric and pentameric CA units (EMD-3465) (12) and cryo-EM density maps of the NPC (EMD-11967) (89). HIV-1 capsid cone model courtesy of Simone Mattei. Abbreviations: CA, capsid protein; CPSF6, cleavage and polyadenylation specificity factor 6; CR, cytoplasmic ring; cryo-EM, cryo-electron microscopy; CypA, cyclophilin A; FG, phenylalanine-glycine; HIV-1, human immunodeficiency virus type 1; IR, inner ring; MT, microtubule; NE, nuclear envelope; NPC, nuclear pore complex; NR, nuclear ring; Nup, nucleoporin.

inter- and intramolecular interactions within intrinsically unstructured FG domains and thereby form the permeability barrier within the central channel of the NPC (see below). The cytosolic protein CypA can interact with the Cyp-binding loop on the capsid surface (114) (**Figure 1*b*,*d***). Upon arrival at the nuclear envelope, cytoplasmic filaments of Nup358 may serve as a landing platform for the capsid (115, 116). Nup358 contains a cyclophilin homology domain, which can bind to the CA Cyp-binding loop (115), as well as FG repeats (present on many Nups) that may interact with the hydrophobic binding cleft. Accordingly, there could be handover of capsids from the cytosolic binding proteins to the cytoplasmic face of the NPC, thereby docking the capsid at the nuclear pore. The observed loss of fluorescently labeled CypA from incoming capsids at the nuclear membrane (86, 94) agrees with the proposed CypA displacement by a competing factor (e.g., Nup358) at this site. ET of HIV-1 capsids at the cytosolic side of the NPC showed that almost all structures detected were oriented with the narrow end pointing toward and entering into the pore (89). Due to the buckminsterfullerene architecture of the cone, the narrow end displays a higher local concentration of curvature-inducing pentamers (**Figure 1*a***), which were suggested to represent preferential binding sites for CypA (117). Preferential interaction of Nup358 with this region could serve to orient the capsid tip toward the NPC, thereby threading the large structure into the narrow channel.

The central NPC channel is not empty but filled with a dense hydrophobic meshwork of intrinsically unstructured FG domains of various Nups (118). It has been proposed that canonical nuclear import involves rapid and selective immersion of the cargo into this unstructured (gel-like) phase (119, 120); the interaction of cargo with the FG meshwork is mediated by the

nuclear import machinery (35). We suggest that the mature HIV-1 capsid lattice with its many FG-binding hydrophobic clefts can act as a multivalent nuclear import factor in a similar way, promoting its own translocation. The HIV-1 capsid may thus not need additional nuclear import factors but may in fact be the nuclear import factor. CA and CA hexamers have been shown by biochemical and structural analyses to interact with FG Nups localized at the cytoplasmic face (Nup88 and Nup214), in the inner channel (Nup62, Nup98, Nup107), and at the nuclear basket (Nup153) (107, 108, 121–125). Once the capsid enters the nuclear pore with its pointed end, sequential binding of FG motifs from central channel and nucleoplasmic Nups to the capsid may drive its immersion into the hydrophobic mesh, similar to canonical nuclear import. Due to the cone shape, the number of exposed hexamers available for FG binding increases from the narrow to the broad end. Thus, an avidity gradient caused by capsid architecture, as well as possibly different affinities of differently located Nups, could provide directionality of the transport. Multivalent binding may overcome the energy barrier to drive translocation of the large structure through the dense protein meshwork (**Figure 3**). The frequent observation of capsids adjacent to the cytoplasmic side or entering the NPC, but rarely inside the central channel (89), indicates that full immersion into the hydrogel-like liquid phase may be rate limiting for capsid nuclear import.

Binding of FG repeats from the nuclear basket protein Nup153 to the capsid lattice (**Figure 1*e***) would then terminate translocation and stall the capsid at the nucleoplasmic side of the pore (**Figure 3*b*,*c***). In the canonical nuclear import pathway, the importin-bound cargo is released from Nup153 by the GTP-bound Ras-related nuclear protein, to enable cargo penetration into the nucleoplasm (35). If the HIV-1 capsid serves as an import factor in a similar way, it would require an analogous release factor. This could be the nuclear protein CPSF6, which comprises an FG motif that binds to the same hydrophobic cleft as Nup153 (**Figure 1*e***) and could thus displace bound Nups. CPSF6 forms large clusters on HIV-1 capsids in the nucleus, and its depletion or the introduction of mutations in CA that interfere with CPSF6 binding arrests incoming capsids at or close to the nuclear pore (81, 83, 85). Initiation of CPSF6 clustering on the incoming capsid tip at the nucleoplasmic side of the pore, as observed by stimulated emission depletion nanoscopy (81, 83), could not only displace Nup153 in a final handover step but also prevent retrograde movement of the capsid into the inner channel. Additional nuclear factors may contribute to release of the capsid from the NPC central channel since CPSF6 knockdown or mutation of CPSF6 binding residues in CA resulted in accumulation of capsid-like structures directly adjacent to the nucleoplasmic side of nuclear pores; these structures apparently had left the central channel but failed to be released further into the nucleoplasm (89). CPSF6 clustering not only serves to release the capsid from the nuclear basket but also directs the capsid-encased subviral complex to nuclear speckles (97, 98, 104), where integration of the viral DNA into the host cell genome occurs (98, 103, 126). Clusters of complete and broken capsid-like structures were often observed by CLEM (99, 100), supporting directed nucleoplasmic trafficking (97, 99, 100). Transportin 1, which has been suggested to bind the CA Cyp-binding loop (87), may also contribute to capsid nuclear import conceivably displacing the Nup358 Cyp homology domain, but this has not been investigated in detail.

In conclusion, we propose the intact capsid to be the elusive nuclear import factor for the HIV-1 replication complex. The described model suggests a testable pathway for capsid nuclear import and provides a mechanistic explanation for the evolution of the cone-shaped capsid.

6. RECENT EVIDENCE FOR NUCLEAR REVERSE TRANSCRIPTION

It is generally agreed that initiation of reverse transcription from the tRNA primer occurs already during HIV-1 formation but is then stalled due to lack of dNTPs and continues once the subviral

complex becomes exposed to the dNTP pool in the cytosol of the newly infected cell (127). While it was initially assumed that the capsid shell must disintegrate to allow access of the RTC to dNTPs after cell entry, the described recent evidence indicates that dNTPs may enter the intact capsid through the R18 pore (75).

Whether reverse transcription is also completed in the cytosol has been more difficult to determine. Through use of imaging-based approaches instead of 2-LTR formation as a readout for RTC/PIC nuclear import, it was demonstrated that reverse transcription is not a prerequisite for nuclear entry of subviral complexes (81, 84, 86, 102). Initial indication that reverse transcription may be completed inside the nucleus came from experiments using incorporation of EdU into newly synthesized HIV-1 cDNA in MDM. Subviral particles containing fluorescently labeled IN exhibited higher EdU intensities inside the nucleus compared to cytoplasmic ones (102). Further evidence for nuclear completion of reverse transcription was derived from inhibitor time-of-addition experiments showing that HIV-1 remains sensitive to RT inhibition beyond nuclear import of subviral complexes (85, 98, 109). Experiments that artificially blocked import through nuclear pores strongly supported this conclusion (109). Releasing nuclear particles from RT inhibition allowed them to resume synthesis of infectious, EdU-containing cDNA (97), and intermediate reverse transcription products were detected in the nuclear fraction upon cell fractionation of infected cells (110). Dharan et al. (109) directly confirmed nuclear reverse transcription by strand-specific hybridization showing that only minus-strand DNA was detected in the cytoplasm, while both minus- and plus-strand DNA were found inside the nucleus (109). The same conclusion was reached employing a fluorescent marker that is specifically recruited to a sequence motif introduced into the viral genome (the ANCHOR system) (99). Binding of this reporter is dsDNA specific, indicating a late product of reverse transcription. A positive signal was observed only on nuclear HIV-1 complexes. Furthermore, nuclear complexes negative for ANCHOR exhibited a twofold lower EdU signal compared to those positive for the signal, again indicating that reverse transcription continues in the nucleus (99). Accordingly, the traditional understanding of RTC that is converted in the cytosol into a (capsid-free) PIC comprising the complete cDNA needs to be revised in light of the described studies.

It is surprising that the central observation of nuclear reverse transcription was only made about 30 years after the first characterization of the RTC. This was most likely caused by the experimental systems used in earlier studies: Cytoplasmic complexes were shown to be integration competent but appeared to be devoid of RT by Western blot (18). They did contain enzymatic RT activity (19), however, and may thus have completed reverse transcription during the reaction. Furthermore, almost all earlier studies used 2-LTR circles as a surrogate marker for nuclear import because their formation requires nuclear factors. However, they also require complete reverse transcription and access of ligase to the cDNA. We suggest that 2-LTR circles do not directly monitor nuclear entry but rather the nuclear presence of complete HIV-1 cDNA that is no longer protected inside the capsid.

It is currently not clear why reverse transcription is not completed in cytosolic subviral complexes, given sufficient dNTP concentration in the cytosol of most cells and the potential of dNTP access to the replication complex through the R18 pore (75, 77). This could be due to nuclear import being faster than reverse transcription. Alternatively, reverse transcription may be stalled by space limitations in the closed environment of the viral capsid, given that b-DNA requires a larger volume than the corresponding, highly branched RNA. This possibility and its consequences for genome uncoating are discussed in Section 7.2. Finally, potential inhibitory cytosolic factors (e.g., blocking the R18 pore), reverse transcription promoting nuclear factors, or *cis*-acting elements in the viral genome may also be involved, and exploring the regulation of reverse transcription will be an important subject of future studies.

7. WHAT IS THE NATURE OF THE NUCLEAR UNCOATING EVENT?

7.1. Uncoating by Physical Rupture of the Capsid Lattice

The molecular mechanism of uncoating could involve either cooperative disassembly of the capsid into individual subunits or breaking of the capsid lattice, thereby creating an opening for genome release. The first model was supported by the observed instability of biochemically isolated HIV-1 capsids in vitro (17–20, 24–26), but rapid capsid dissociation was later explained by lack of IP6 as discussed above (73, 74); addition of IP6 during preparation allowed isolation of stable cone-shaped HIV-1 capsids (128). Enhanced capsid stability due to IP6 in the cytosol does not rule out capsid dissociation as the mechanism of uncoating, however. A cooperative disassembly model was supported by the observation that a GFP-CA fusion protein used to label HIV-1 capsids in infected cells was completely lost shortly before chromosomal integration (85). However, the same study also revealed rapid loss of the GFP-CA signal upon addition of PF74. This compound displaces CPSF6 from nuclear subviral complexes but actually revealed the previously shielded underlying CA in immunofluorescence experiments rather than dissolving it (see Section 4.1) (99). Furthermore, broken capsid-like structures were visualized by negative-stain EM and cryo-ET upon addition of the related GS-CA1 inhibitor targeting the same CA region (128). GFP-CA is incorporated in substoichiometric amounts into the viral particle and could be lost more rapidly from the subviral complex than wild-type CA due to less stable association with the capsid lattice. However, this does not explain the concomitant loss of CPSF6 from nuclear subviral structures in another study by some of the same authors (62), which suggests disassembly of the capsid lattice.

Evidence for capsid rupture rather than cooperative disassembly as an uncoating pathway mostly came from CLEM and ET of cells infected with a virus carrying a fluorescent replication protein and/or the ANCHOR system for detection of accessible HIV-1 cDNA (89, 99, 100). In these studies, ruptured capsid-reminiscent structures and incomplete capsids, lacking internal electron density, were commonly found at nuclear positions that were identified by the fluorescent replication protein (**Figure 4*c***); lack of internal density indicated that they may represent remnants of the capsid shell after genome uncoating (89, 99). Similar broken structures had been reported previously upon endogenous reverse transcription in vitro in isolated HIV-1 cores (128) (**Figure 4*b***). Nuclear capsid rupture with subsequent dissociation of the genome from the broken structure was further supported by fluorescence imaging of infected cells, showing spatial separation of specific viral DNA signals from the bulk of replication proteins in the nucleus at later time points after infection (81, 82, 96–99). Through use of the ANCHOR system, separation of viral dsDNA from a fluorescent marker protein identifying the capsid remnant could be directly observed by live imaging (99, 129).

Taken together, the available evidence indicates completion of HIV-1 reverse transcription in the nucleus, concomitant with or followed by breach of capsid integrity and physical separation from the genome with associated factors. The observation of broken capsid remnants argues against cooperative disassembly, while rapid loss of fluorescent GFP-CA and CPSF6 suggests the opposite, and further studies are needed to resolve this question.

7.2. What Are the Trigger and Mechanism of HIV-1 Genome Uncoating?

A link between reverse transcription and uncoating dynamics was already suggested by early analyses studying the effect of CA mutations and RT inhibition on the outcome of infection (27–33). More recent experiments in MDM revealed that increasing the intracellular dNTP pool by depletion of the deoxynucleoside triphosphohydrolase SAM domain and HD domain-containing protein 1 enhanced the rate of reverse transcription (96, 102) and led to faster separation between

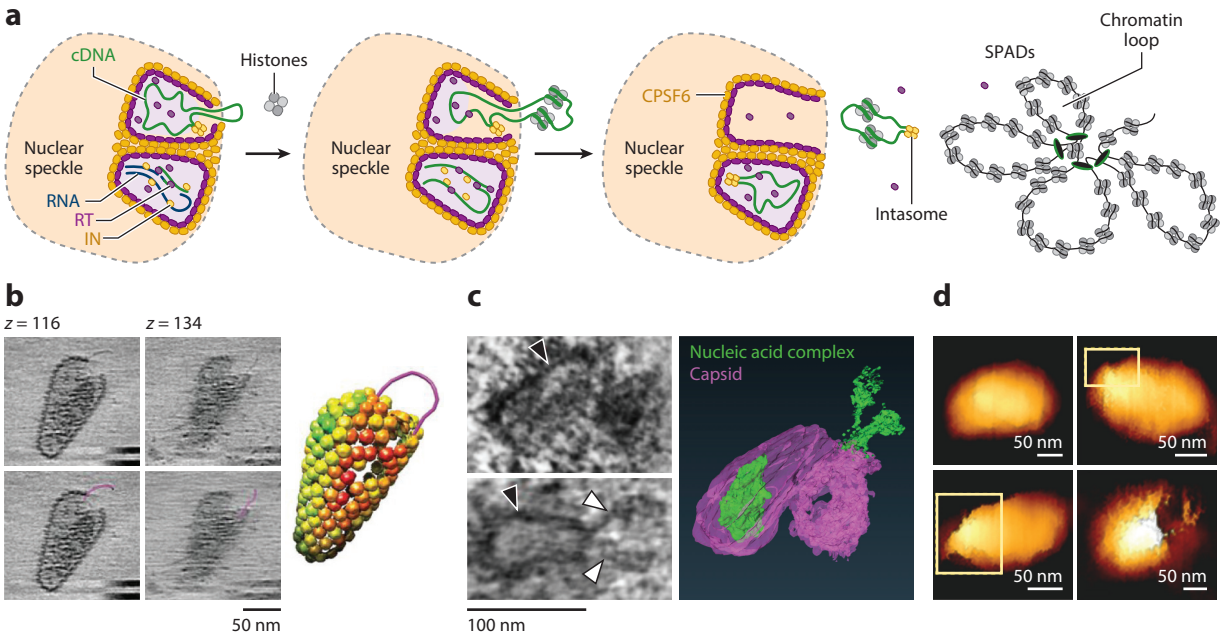

Figure 4

A model for the mechanism of nuclear uncoating. (*a*) Scheme. Reverse transcription is finished inside the nucleus, likely within nuclear speckles. The spatial demand of the newly synthesized dsDNA may induce cracking of the capsid. Protruding cDNA loops can become exposed to the nuclear environment. Chromatinization and/or other nuclear factors likely facilitate complete rupturing of the lattice to allow full uncoating of the integration-competent dsDNA. The viral genome is then integrated within SPADs of the chromatin in close proximity to the uncoating site. (*b*) In vitro reconstitution experiments. Efficient endogenous reverse transcription was induced in isolated cores by addition of dNTPs in the presence of IP6. As a result, an opening with an emanating polynucleotide loop was visualized using cryo-electron tomography. Z sections (*left*) and a 3D rendering (*right*) of the hexamer and loop arrangement are shown. The hexamers are color coded by cross correlation determined through subtomogram averaging from high (*green*) to low (*red*) correlations. Panel *b* adapted from Reference 128 with permission. (*c*) Visualization of capsid-like structures inside the nucleus of infected cells using correlative light and electron microscopy and electron tomographic analysis. Reconstructed electron tomograms were correlated to a position positive for both a fluorescently tagged IN fusion protein and newly synthesized HIV-1 dsDNA exposed to the ANCHOR system. A cluster of conically shaped structures (*black arrowheads*) was observed. While some structures displayed internal electron density indicating the presence of the nucleic acid complex (*top left subpanel*), another lacked internal density but was connected to a dense structure protruding from the narrow end (*bottom left subpanel*, *white arrowheads*), consistent in appearance with chromatinized DNA. The left subpanels show different cross sections, and the right subpanel shows a 3D rendering of the same position. Panel *c* adapted from Reference 99 (CC BY 4.0). (*d*) Isolated IP6-stabilized capsids were subjected to endogenous reverse transcription reactions and analyzed using atomic force microscopy. The top left subpanel shows a representative capsid prior to dNTP addition, while the other subpanels display capsids with smaller ruptures (*yellow boxes*) and large openings (*bottom right subpanel*) 5 h after induction of endogenous reverse transcription. Panel *d* adapted from Reference 77 (CC BY 4.0). Abbreviations: cDNA, complementary DNA; CPSF6, cleavage and polyadenylation specificity factor 6; dNTP, deoxynucleoside triphosphate; dsDNA, double-stranded DNA; IN, integrase; IP6, inositol hexakisphosphate; RT, reverse transcriptase; SPAD, speckle-associated domain.

EdU and CA signals inside the nucleus (96), implicating progression of reverse transcription in genome uncoating. Theoretically, increased spatial demand of the viral dsDNA compared to the single-stranded RNA (ssRNA) genome may breach capsid integrity by mechanical force. Such a cDNA confinement-induced uncoating model was proposed by Rouzina & Bruinsma (130). dsDNA is a relatively rigid polymer with a persistence length p of ~50 nm (131), while ssRNA has a p of ~1 nm (132). Estimating the radius of gyration Rg (133) as a measure for the volume requirement for the ideal polymer (nucleotide string without secondary structure) with a contour

length L of ~0.5 nm per nucleotide with

$$Rg \cong (L^* p)^{0.5}$$

results for a genome of 10,000 bases (or base pairs) in an Rg of ~70 nm for ssRNA and ~500 nm for dsDNA. This difference together with the extensive branching of ssRNA (134) may explain how two 10 kb ssRNA molecules fit into a single HIV-1 capsid with minimal confinement free energy costs, while the product of reverse transcription may not. Fitting unbranched and uncondensed b-DNA of this size into the available space of the HIV-1 capsid would most likely exert a disruptive outward force that could cause rupture of the capsid lattice. However, the in silico model (130) also predicts that compaction of HIV-1 nucleic acids by the viral nucleocapsid (NC) protein, present within the core at high concentration, strongly alleviates outward forces acting on the capsid shell, and NC-mediated condensation of DNA into a tightly packed globule has been demonstrated in vitro (135). The limit of dsDNA length that can be accommodated within the HIV-1 capsid under authentic conditions is currently not known.

The genome size compatible with HIV-1 replication is limited, and larger artificial insertions result in loss of infectivity or subsequent deletions during replication. Whether premature disruption of the capsid could play a role in the replication defect caused by increased genome size is unknown, as the defect may already occur at the packaging and assembly step. However, lentiviral vectors packaging RNA much smaller than 10,000 nucleotides have been shown to efficiently transduce cells and are thus capable of genome uncoating and integration. This was generally observed under steady-state conditions, however, and a potential effect of genome length on the kinetics and efficiency of genome uncoating has not been investigated so far. Accordingly, the influence of genome size on uncoating, together with the NC-mediated compaction, will be an important aspect of further studies.

Experimental support for capsid rupture and genome uncoating by separation, as well as for the confinement-driven uncoating hypothesis, was recently provided by the elegant in vitro study by Christensen et al. (128). These authors isolated HIV-1 capsids from purified virions in the presence of IP6 and performed endogenous reverse transcription in vitro by addition of dNTPs followed by cryo-ET (128). Strikingly, newly synthesized cDNA remained associated with incomplete capsid-like structures with different degrees of opening, and DNA-like loops often emanated from small openings in the lattice (128) (**Figure 4*b***). These structures were reminiscent of subviral structures observed in the nucleus of infected cells by CLEM and ET (89, 99) (**Figure 4*c***), in which electron-dense elongated structures protruded from the opening of an empty capsid-like object. Such elongated objects were also found separated from the viral proteins at later time points after infection and colocalized with the ANCHOR marker, indicating that they comprise viral dsDNA (99). In the cellular context, the structures presumed to represent dsDNA were more electron dense compared to the respective structures resulting from endogenous reverse transcription in vitro. The former structures resembled chromatinized DNA, and we hypothesize that full uncoating of the genome may require chromatinization of emerging DNA and possibly other nuclear factors. Rapid chromatinization has been observed for retroviral genomes when they become accessible in the nucleus of the infected cell (136–139). Integration of the products of endogenous reverse transcription using isolated HIV-1 capsids required the presence of nuclear extracts in addition (128), and it will be interesting to analyze whether full uncoating and (partial) chromatinization may occur in this case.

Atomic force microscopy (AFM) on isolated HIV-1 capsids undergoing endogenous reverse transcription supports the confinement-driven uncoating hypothesis (30, 77, 140, 141). In the presence of IP6, multiple stiffness peaks together with morphological changes of the capsid were observed and were interpreted as strand transfer events during reverse transcription (141). At later

time points, ruptured capsid structures were visualized by AFM (30, 77) (**Figure 4*d***), similar to the findings from ET (89, 99, 100, 128). From these results, it was concluded that cDNA synthesis inside the capsid exerts a detectable outward force and that the capsid has some flexibility to accommodate this spatially demanding process but will eventually break.

While these results indicate a role of cDNA completion in capsid rupture and genome uncoating in the nucleus, other factors may contribute as well. Conceivably, passage of the large capsid through the central NPC channel may lead to physical damage, with subsequent stabilization through CPSF6 coating. This may render the structure more sensitive for rupture upon cDNA completion in the nucleus. Alternatively, the capsid may encounter uncoating factors upon reaching its destination in or near nuclear speckles.

8. CONCLUSION, SYNTHESIS, AND THERAPEUTIC EXPLOITATION

Taken together, the described studies indicate that the cytosolic post-fusion capsid engages with microtubule-associated proteins, transporting it toward the nuclear region. Reverse transcription commences during cytosolic trafficking but does not achieve completion before nuclear entry. Morphologically complete capsids enter nuclear pores with their narrow end first and can translocate through the central channel of dilated pores. Once reaching the nuclear basket, the capsid lattice interacts with CPSF6, transporting the complex to nuclear speckles, where genome uncoating and integration occur. The mechanism of capsid rupture and genome uncoating is currently not known, but it may involve the completion of cDNA synthesis. In conclusion, the HIV-1 capsid appears to be the central orchestrator of early post-entry replication (142). This involves at least five important roles:

1. Facilitating cytoplasmic transport processes: The capsid serves as a cytosolic transport module to direct the subviral post-fusion complex from the plasma membrane to the nuclear envelope. This employs microtubule-mediated transport by tethering kinesin and dynein molecular motors via specific recruitment of adapter proteins to the hexameric capsid lattice.
2. Protecting the viral replication machinery: The closed capsid shell prevents degradation of nucleic acid by cellular nucleases and innate immune activation by DNA sensors that would result in cell death and induction of an antiviral state.
3. Enabling efficient reverse transcription: Confinement of the replication complex within the capsid supports reverse transcription by preventing the loss of replication factors and by potentially concentrating dNTPs through the R18 pore. The closed environment further facilitates efficient strand transfer and recombination.
4. Serving as unusual nuclear import receptor: The hexameric lattice of the capsid may constitute a multimeric nuclear import receptor, engaging various Nups through its interaction surfaces. Capsid size appears to be at the upper limit even of dilated nuclear pores, and evolution of the conical shape may be essential for import of such large cargo; it could facilitate threading the capsid into the central channel and may provide an avidity gradient for immersion into the gel-like transmission barrier.
5. Facilitating nucleoplasmic transport to specific subnuclear regions: Coating the capsid lattice with CPSF6 upon nuclear entry leads to targeting of the subviral structure to nuclear speckles, where genome uncoating and integration occur. Clustering of multiple subviral structures at these speckles suggests common trafficking routes with HIV-1 genome integration commonly occurring in transcriptionally active speckle-associated domains.

CA has long been considered a good target for inhibitor development but mainly based on the important role of the CA domain in assembly of the immature virus. The described newer results

indicate, however, that CA has a second essential function in the early phase of replication, and CA-directed inhibitors may thus block HIV-1 replication prior to genome integration. Accordingly, several recently developed CA-targeting inhibitors have been shown to interfere with HIV-1 early replication, commonly targeting the FG-repeat binding pocket that is essential for host factor interaction (143–145). This includes compounds PF74 (146), GS-CA1 (147), and most notably GS-6207 (lenacapavir) (148, 149), the latter currently in clinical phase II/III trials. Lenacapavir is active in the low nanomolar range and can serve as a long-acting drug requiring administration only every few months (148). All three compounds contain a central phenyl ring superimposing with the F residue of FG repeats in host factor binding peptides and compete for the respective FG repeat factors. Accordingly, they affect nuclear import and consequently nuclear completion of reverse transcription, thereby blocking HIV-1 replication before integration can occur. Small-molecule leads targeting the central R18 pore could also be developed, e.g., on the basis of hexacarboxybenzene, which can block dNTP import and reverse transcription (75). Similarly, small molecules may be envisaged that could displace IP6 or by themselves have a propulsive effect on the R18 pore, thereby leading to premature disassembly of the capsid. Clearly, given the multiple nonredundant roles of the capsid in HIV-1 replication, most importantly in the early phase, interfering with capsid integrity or interaction with host components is an ideal target for novel antivirals.

FUTURE ISSUES

1. What is the molecular mechanism of nuclear import of the human immunodeficiency virus type 1 (HIV-1) capsid—can the handover model be confirmed?
2. Are there common trafficking routes and destination pathways for the HIV-1 subviral complex?
3. What is the trigger for HIV-1 genome nuclear uncoating—does completion of reverse transcription play a role?
4. How is the HIV-1 genome released from the ruptured capsid—is there a role for chromatinization or other nuclear factors?
5. What distinguishes integration into transcriptionally active versus silent sites—can this information be useful for eventually overcoming latency?

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