Virus Research

Review

Multiple roles of the capsid protein in the early steps of HIV-1 infection

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ABSTRACT

The early steps of HIV-1 infection starting after virus entry into cells up to integration of its genome into host chromosomes are poorly understood. From seminal work showing that HIV-1 and oncoretroviruses follow different steps in the early stages post-entry, significant advances have been made in recent years and an important role for the HIV-1 capsid (CA) protein, the constituent of the viral core, has emerged. CA appears to orchestrate several events, such as virus uncoating, recognition by restriction factors and the innate immune system. It also plays a role in nuclear import and integration of HIV-1 and has become a novel target for antiretroviral drugs. Here we describe the different functions of CA and how they may be integrated into one or more coherent models that illuminate the early events in HIV-1 infection and their relations with the host cell.

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Contents

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS or acquired immunodeficiency syndrome. It is a lentivirus uniquely adapted to replicate in human cells, in particular CD4+ cells such as helper T-lymphocytes, macrophages and microglial cells. HIV-1 tropism depends mainly on the expression of the cell receptor CD4 and co-receptors CCR5 and CXCR4. The seven-transmembrane domain chemokine receptor CCR5 is mainly present on the surface of memory CD4+ T-cells, macrophages

and microglial cells, whereas the CXCR4 co-receptor is mainly expressed in naïve CD4+ T-cells (Freed and Martin, 2001).

The HIV-1 genome in its integrated DNA form is approximately 9.8 Kb in size and encodes for three polyproteins (Gag, Gag–Pol and Env) and six smaller accessory proteins (Vif, Vpr, Vpu, Tat, Rev and Nef). Env mRNA is spliced and translated into gp160, which is then cleaved by a furin-like cellular protease into a "surface" (SU also called gp120) and a "transmembrane" region (TR also called gp41). Gp120 is responsible for engaging with CD4 and the co-receptors, whereas gp41 induces fusion of the viral membrane with the cell membrane. Pol encodes for the viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN). Gag encodes for structural proteins matrix (p17 MA), capsid (p24 CA), nucleocapsid (p7 NC), p6 and spacer peptides Sp1 and Sp2 (Freed and Martin, 2001). Gag forms the capsid core and during or shortly after budding the viral protease cleaves it into the different

components inducing dramatic conformational rearrangements that have important functional consequences (Bharat et al., 2012).

In the unprocessed form, Gag polyproteins form a capsid core of approximately 100 nm diameter with a regular arrangement whereby p17 MA is at the outer region in direct contact with the viral membrane, followed by p24 CA and NC, which is central and contacts the viral RNA genome (Bharat et al., 2012; Briggs and Krausslich, 2011; Yeager et al., 1998). Following protease activation and Gag cleavage, the immature core undergoes a dramatic rearrangement resulting in a fullerene conical structure of 100–120 nm in length and 50–60 nm wide composed of approximately 250 CA hexameric rings organized in a lattice (Fig. 1) (Briggs et al., 2003; Ganser et al., 1999; Ganser-Pornillos et al., 2007). Importantly, the presence of 12 CA pentamers distributed at the edges of the cone, 7 at the wide end and 5 and the narrow end, allows the lattice to curve and assume the typical conical geometry (Pornillos et al., 2011). The CA subunit within the hexameric ring are stabilized by quite extensive N-terminal domain (NTD)–NTD intermolecular interactions and less extensive NTD–CTD intermolecular interactions, mainly between NTD helices 4 and 7 and CTD helices 8 and 11, whereas adjacent rings are linked together mainly by mobile C-terminal domain (CTD)–CTD intermolecular interactions (Pornillos et al., 2009) (Fig. 1). The mobility of the CTD–CTD interactions allows the formation of the curvature in the lattice (Pornillos et al., 2009). Some details of how the conical geometry is generated are still unknown because high resolution X-ray crystallography cannot be applied to an intact core.

CA forms the building block of the hemaxers in the lattice. It is a 24 kDa protein with independently folded NTD and a CTD that are flexibly linked (Berthet-Colominas et al., 1999; Gamble et al., 1997; Gitti et al., 1996; Momany et al., 1996). The structure is highly helical; the NTD contains 7 alpha helices and the CTD contains 4 (Gamble et al., 1997; Gitti et al., 1996). Furthermore CA has a large loop encompassing residues 85–93 between helices 4 and 5 that is exposed to the surface of the hexamer and binds cyclophilin A (CypA) (Gamble et al., 1996) (Fig. 2).

There are approximately 1500 CA monomers in a mature HIV-1 core but there appears to be an excess of Gag molecules in the immature core (up to 5000) (Briggs et al., 2004). Upon Gag cleavage, some CA molecules are not incorporated into the mature core but the function of the excess CA is not known (Briggs et al., 2004). The mature retroviral core is less stable than the immature form and it has been difficult to isolate intact HIV-1 cores to perform biochemical and genetic studies. However it is possible to purify small amounts of mature HIV-1 cores by sedimentation in sucrose gradients through a thin layer of mild detergent, which strips the envelope and matrix (Aiken, 2009). Alternatively, many aspects of the core structure and function can be studied in vitro upon formation of the so-called "tubes". These structures are generated by incubating purified recombinant CA or CA–NC with short RNAs or single stranded DNA oligonucleotides in the appropriate salt conditions (Ganser et al., 1999; Gross et al., 1998). The introduction of the point mutation R18A in CA helps generating lattices of different geometry, including spheres, cones and cylinders, which recapitulate the bonafide core structure (Ganser-Pornillos et al., 2007).

2. CA, core uncoating and reverse transcription

Following cell-receptor mediated entry, HIV-1 starts reverse transcribing its RNA genome. An early function of HIV-1 CA is to provide a suitable environment for reverse transcription within the so-called reverse transcription complex (RTC). The virus must also shed its core or "uncoat" to progress through the various steps of the life cycle. We have an incomplete understanding of these early steps, however some progress has been made in recent

years. Biochemical fractionation in sucrose gradients of the cytosol of acutely infected cells indicated that uncoating happens quite early post-infection, probably within 1 h in the case of HIV-1 but only after nuclear entry following mitosis in the case of murine leukemia virus (MLV) (Fassati and Goff, 1999, 2001; Karageorgos et al., 1993). Notably, the results of the biochemical fractionation used to characterize MLV RTCs have been recently confirmed using a combination of elegant imaging and genetic approaches (Prizan-Ravid et al., 2010). Because the same fractionation procedure was used to characterize both viruses, the simplest explanation for the different time of uncoating of HIV-1 and MLV is that the MLV core is more stable than the HIV-1 core in the cytoplasm of infected cells. This also explains, at least in part, why MLV cannot infect non dividing cells, given that large amounts of CA are still associated with its RTC and pre-integration complex (PIC), presumably making it too bulky to go across nuclear pores (NPCs) (Bowerman et al., 1989; Fassati and Goff, 1999). The instability of the HIV-1 core as determined in biochemical assays may be less pronounced in the cytoplasm of infected cells, where host factors could stabilize it. However recent genetic evidence supports the idea that HIV-1 uncoating happens as early as 30–45 min post-infection (Hulme et al., 2011; Perez-Caballero et al., 2005). In fact the time of uncoating seems to be cell-type dependent, faster in HeLa cells, slower in CD4+ T-lymphocytes, suggesting that host cells factors may play a role as well (Arfi et al., 2009). There is evidence supporting the idea that significant uncoating happens before reverse transcription is completed. APOBEC3G is a restriction factor for HIV-1, counteracted by the viral accessory protein Vif, that promotes G to A mutations by deaminating C to U on the negative sense, single stranded viral DNA generated during reverse transcription (Malim, 2009). Recently, it has been shown that APOBEC3G in the target cells can attack the incoming virus in macrophages, suggesting that the protein has access to the viral genome before the completion of reverse transcription (Koning et al., 2011). LEDGF/p75 is a host co-factor that binds to HIV-1 IN and stimulates targeted integration into the host genome (Engelman and Cherepanov, 2008). A dominant form of LEDGF/p75 that localizes exclusively in the cytoplasm can target IN and perturb integration, suggesting that IN is accessible within the RTC/PIC (Meehan et al., 2011). Several host cell factors have been implicated in promoting reverse transcription, including nucleic acids binding proteins, DNA repair and splicing factors (Konig et al., 2008) and it is not unreasonable to assume that at least some of them act in the target cells on the incoming RTC, which must therefore be accessible. More recent data demonstrates that uncoating and reverse transcription proceed in parallel and may influence each other (Arfi et al., 2009; Hulme et al., 2011). It also not clear how much CA is shed from the core in the cytoplasm. Biochemical studies found that most CA is shed from the HIV-1 RTC and PIC (Bukrinsky et al., 1993; Farnet and Haseltine, 1991; Fassati and Goff, 2001; Karageorgos et al., 1993; Miller et al., 1997), yet it is quite possible that host factors compensate in part for the intrinsic instability of the HIV-1 core inside cells and that in fact more CA remains associated with the RTC than previously thought.

If uncoating happens too quickly however, reverse transcription does not take place. This is supported by two lines of evidence. TRIM5α is a restriction factor that blocks HIV-1 infection by targeting the incoming capsid core and inducing its proteasomal degradation (Malim and Bieniasz, 2012). TRIM5α acts very early post-infection (<1 h) and causes premature uncoating, leading to aborted reverse transcription (Malim and Bieniasz, 2012; Roa et al., 2012; Stremlau et al., 2006). Second, mutations in CA that make the HIV-1 core unstable also cause premature uncoating and aborted reverse transcription (Forshey et al., 2002). In fact there is elegant work based on specific mutations in CA and their functional characterization showing that optimal stability of the HIV-1 core is important for reverse transcription and for later
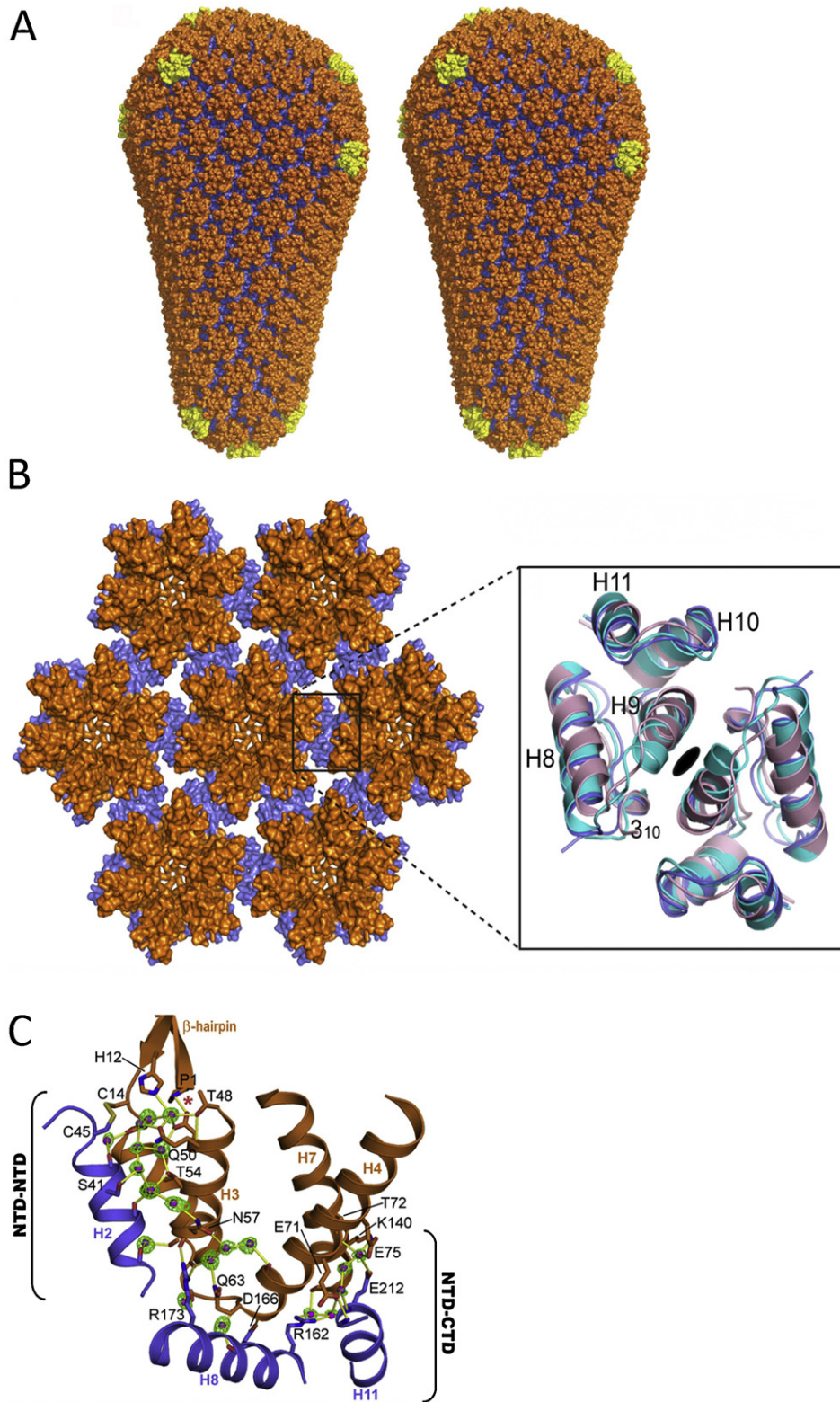
Fig. 1. Structure of the HIV-1 core and the CA hexameric lattice. (A) Stereo view of a backbone-only fullerene cone model composed of 1056 CA subunits. The hexamers, pentamers and dimers are coloured in orange, yellow and blue, respectively. Reprinted from Pornillos et al. (2011) page 426 by permission from McMillan Publishers Ltd. (B) Top view of one sheet in the templated CA crystals, which recapitulates the hexameric lattice of authentic capsids at its planar limit. The NTDs are colored orange, and the CTDs are blue. Interactions between neighboring hexamers are mediated only by the CTD. Right hand box, top view of the CTD–CTD interface that connects neighboring hexamers. The black oval represents the twofold symmetry axis. Reprinted from Pornillos et al. (2009) page 1282 with permission from Elsevier. (C) Polar and water-mediated intermolecular CA contacts within a hexamer. Selected side chains are shown and labeled. Green mesh shows unbiased FO-FC density contoured at +3 s. These were modeled as water molecules (magenta spheres) in the structure derived from hexagonal crystals. Putative hydrogen bonds are represented by yellow lines. Reprinted from Pornillos et al. (2009) page 1286 with permission from Elsevier.
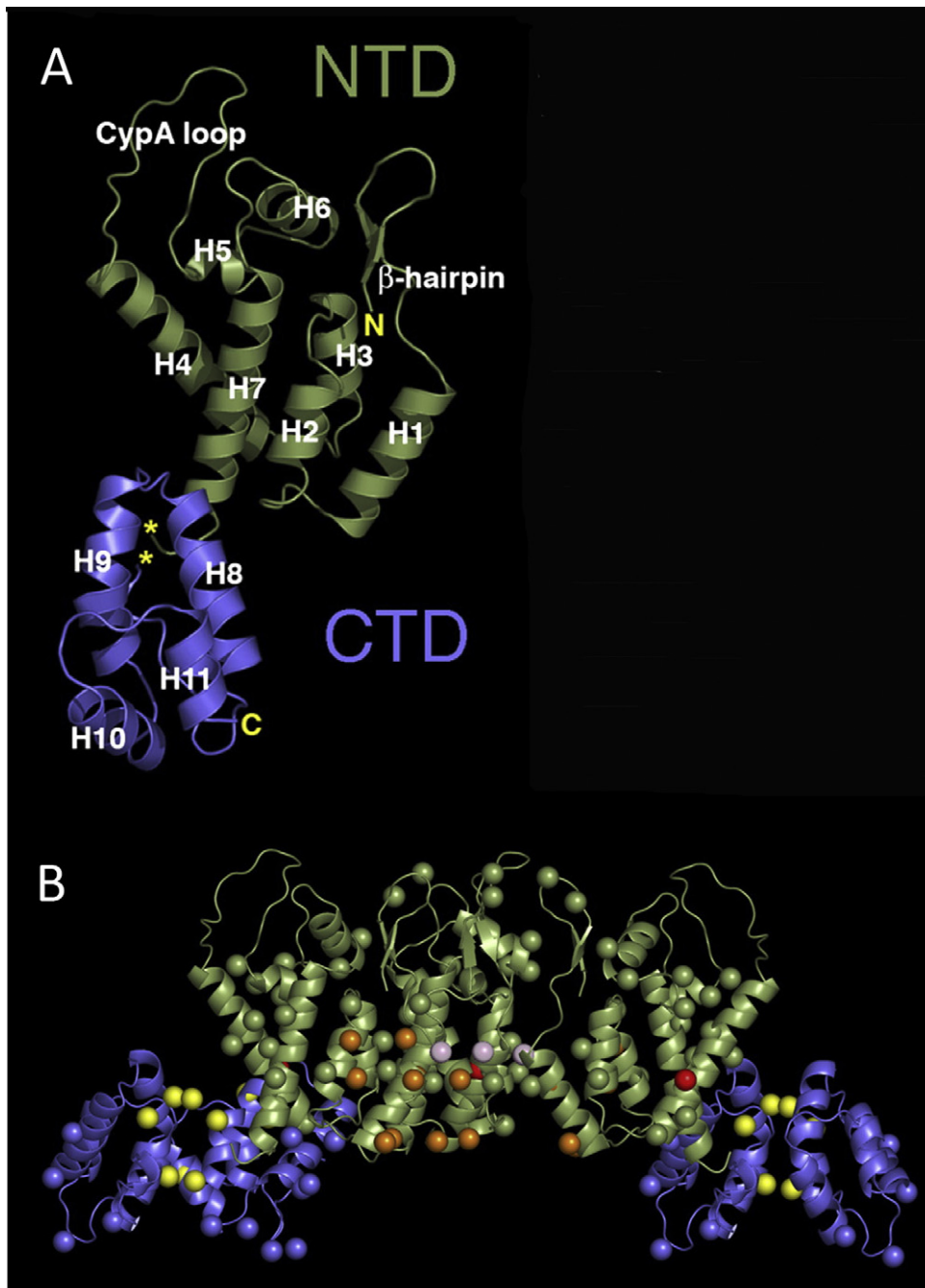
Fig. 2. Structure of the CA protein. (A) Side view of the pseudoatomic monomer model of full-length CA, with the secondary structural elements labeled, including the cypA-binding loop. The carboxy terminus of the NTD and amino terminus of the CTD (approximately 4 Å apart) are indicated by yellow asterisks. (B) Slabbed side view of one hexamer, plus CTDs from adjacent hexamers. Coloured dots indicate mutations that affect core assembly. Note the position of the cypA binding loop facing outwards. Reprinted from Ganser-Pornillos et al. (2007) page 131 with permission from Elsevier.

events, including nuclear transport and integration (Dismuke and Aiken, 2006; Forshey et al., 2002; Yamashita et al., 2007). Therefore it appears that the viral core must remain more or less intact for a short period of time, presumably to allow the early steps of reverse transcription (Roa et al., 2012).

If significant amounts of CA molecules are shed during reverse transcription, how are the various components of the RTC kept together in a suitable structure where DNA synthesis can take place? Recently it has been shown that eukaryotic elongation factors 1A and 1G (EIF1A and EIF1G) associate with RT and IN and may stabilize the RTC, stimulating the late steps of reverse transcription (Warren et al., 2012). The same report and a separate global

proteomics analysis of host factors interacting with HIV-1 proteins identified several EIF3 family members that bind to Pol (Jager et al., 2012; Warren et al., 2012), although in the case of EIF3D the binding resulted in inhibition of reverse transcription, and in a different study overexpression of EIF3F or its truncated form was shown to interfere with viral mRNA production (Jager et al., 2012; Valente et al., 2009).

Uncoating can also occur at a later stage, once reverse transcription is completed. For example, one electron microscopy study detected intact cores several hours post-infection and presumably those cores had completed DNA synthesis (Arhel et al., 2007). Indeed the synthesis of the so-called central polypurine tract (cPPT)

(a triple DNA strand being synthesized late during reverse transcription) appeared to stimulate uncoating (Arhel et al., 2007), although there is controversy on the precise role of the cPPT in HIV-1 infection (Fassati, 2006). Similar to the biochemical approaches, imaging approaches also suffer from the caveat that it is often unclear which form of the RTC is functional, particularly when cells have been infected at very high multiplicity of infection (MOI). It is therefore important to combine multiple approaches or perform comparative analyses of different viruses and selected mutants when studying retroviral uncoating (Arhel, 2010; Hulme et al., 2011).

It is presently unclear where uncoating takes place within cells; some evidence suggests it occurs in the cytoplasm (Hulme et al., 2011; McDonald et al., 2002), other evidence suggests at NPCs (Arhel et al., 2007). Presumably it can happen in both compartments and the question is what selective advantage the virus can gain by uncoating in one cellular location relative to another. Uncoating at the NPC might reduce exposure of the viral RNA/DNA to the cytosolic environment and hence reduce the chances of its degradation. Yet purified RTCs containing little CA appear to be resistant to nuclease attack (Fassati and Goff, 2001; Nermut and Fassati, 2003), suggesting that the virus has evolved ways to protect its genome in the cytoplasm of infected cells. Another possibility is that a shorter exposure to the cytosolic environment by uncoating at the NPC may lessen the risk of activation of the innate immunity pathways sensing cytosolic RNAs or DNAs (Schaller et al., 2011). Although this idea is attractive, it does not explain why the majority of RTCs that uncoat in the cytoplasm fail to activate the innate immune response. On the other hand, a rapid loss of CA and core disassembly may in fact protect the virus from recognition by the innate immune system and restriction factors that target CA (Pertel et al., 2011; Towers et al., 2003).

Therefore the role of CA in these early events is critical. It facilitates reverse transcription by controlling core stability, it may be important to modulate recognition by the innate and intrinsic immune system and to recruit important host cell factors, such as CypA.

3. CA and CypA

To my knowledge, the first indirect evidence that CypA was involved in HIV-1 infection was published in 1988 by Wainberg et al., who showed that the drug cyclosporine A (CsA), a compound that binds to CypA and has potent immunosuppressive activity, prevented acute HIV-1 infection. Interestingly, the authors made the following observations. First, CsA acted on the early steps of infection. Second, chronically infected cells were not sensitive to the drug. Third, CsA was more active in CD4+ T-cells than macrophages. Although the authors interpreted the effects of CsA as inhibition of viral entry, they provided an important seminal observation.

The first demonstration that CypA and CypB bind to the CA region within HIV-1 Gag was provided by Luban et al. (1993) using a yeast two hybrid screen and shortly afterwards CypA was found to be specifically incorporated into HIV-1 but not in simian immunodeficiency virus (SIVmac) and to be important for infection (Franke et al., 1994; Thali et al., 1994). More recent data indicate that the interaction between CA and CypA is widespread among lentiviruses, with the exception of SIVmac, and in fact probably predates HIV by 12 million years, arguing in favor of an important and evolutionary conserved role (Goldstone et al., 2010). Of the various cyclophilins family members, CypA is important for HIV-1 infection, as demonstrated by knock out studies in human CD4+ T-cells (Braaten and Luban, 2001). Although initially it was thought that CypA present in the producer cells and incorporated into budding

viruses was critical for infection, later studies indicated that CypA in target cells is more important, which is now the consensus in the field (Hatziioannou et al., 2005; Sokolskaja et al., 2004). X-ray crystallography revealed that CypA binds to a loop in CA encompassing residues 85–93 between helices 4 and 5, with residues G89 and P90 being critical for binding (Gamble et al., 1996). Importantly, when the CA subunits are organized in a hexamer like in the native core, the CypA-binding loop is exposed outwards on the surface, which explains how CypA in target cells can bind to the incoming virus (Gamble et al., 1996; Pornillos et al., 2011) (Fig. 2).

Despite intensive investigation over nearly 20 years, the precise function of CypA in HIV-1 infection is still not known. CsA is known to inhibit competitively CypA binding to CA and to impair the early steps of HIV-1 infection, before or at reverse transcription (Braaten et al., 1996b; Ptak et al., 2008; Rosenwirth et al., 1994) but the mechanism is unclear. One possibility is that CypA promotes HIV-1 uncoating by destabilizing the viral core. CypA is a peptidyl prolyl cis/trans isomerase (PPIase) that catalyses the slow cis–trans isomerization of proline peptide (Xaa-Pro) bonds (Fischer et al., 1989). Therefore it was hypothesized that CypA, by isomerization of the G89-P90 bond, could change the conformation of the loop and destabilize the core. CypA can indeed isomerize the G89–P90 bond from cis to trans and CypA acetylation inhibits the efficiency of the reaction (Bosco et al., 2002; Lammers et al., 2010), stabilizing the bond in trans. However it is unclear what effects the isomerization of the G89–P90 bond may have. The co-crystal structure of CypA and CA and that of the hexameric CA ring do not provide any obvious mechanism by which subtle changes in the loop conformation may affect the stability of the core (Gamble et al., 1996).

An alternative hypothesis is that CypA, by binding to the loop, may sterically inhibit the CA–CA interactions within the hexameric lattice forming the core. If the ratio of CA to CypA is high enough (close to 1:1), then CA hexamers separate completely, which would lead to the collapse of the core (Gamble et al., 1996). It is therefore interesting to note that at a sufficiently high concentration, Cyp effectively disassembles CA–NC tubes formed in vitro but has little or no effect at lower concentrations (ratio of Cyp to CA >1:10) (Grattinger et al., 1999; Wiegers et al., 1999). Therefore one can envisage a scenario whereby CypA progressively binds the incoming viral core over time, until a critical point is reached (local ratio of CypA to CA close to 1:1), CA subunits are sterically separated and parts of the core collapse. Conversely, cis to trans isomerization of the G89–P90 peptide might stabilize rather than de-stabilize the core, hence CypA might exert two opposing effects on the core; at low concentrations (in the very early stages post-entry), CypA may stabilize the core by promoting the cis isomerization of the peptidyl bond but at critically high concentration it might eventually induce uncoating. According to this model, those areas of the core that bound saturating levels of CypA would collapse whereas other areas that bound less CypA would remain intact, ensuring a progressive rather than a one-step uncoating event.

This scenario would explain the opposite effects of CsA observed on core stability and the differences in the time of uncoating observed in different cell types, which express different levels of CypA (Arfi et al., 2009; Hatziioannou et al., 2005; Li et al., 2009; Matsuoka et al., 2009). Furthermore, stabilization and de-stabilization of the HIV-1 core by CypA may dictate the degree of susceptibility to restriction factors that target incoming CA such as TRIM5α and TRIMCyp (Towers, 2007). Interestingly, whereas mutations of residues G89 and P90 determine a loss of CypA binding to CA and compromise HIV-1 infectivity (Bukovsky et al., 1997), other mutations in CA do not affect CypA binding yet make the virus insensitive to CsA, and some mutations even make the virus dependent on CsA for replication in a cell-type dependent way (Aberham et al., 1996; Braaten et al., 1996a; Yang and Aiken, 2007; Yin et al., 1998). This suggests that CA may be able to compensate for the

loss of CypA binding by modulating the inter-molecular interactions that stabilize the hexameric lattice. Alternatively, some CA mutants might have evolved the ability to exploit cellular factors other than CypA to the same end. Because CA–CypA interactions may be critical for modulating the dynamics of core uncoating, they are likely to influence downstream events of the HIV-1 life cycle.

4. CA and nuclear import

Lentiviruses such as HIV-1 can infect non-dividing cells, in contrast to oncoretroviruses (such as MLV), which require disassembly of the nuclear envelope during mitosis for productive infection. This physiological property of HIV-1 is important for pathogenesis, given that the virus can infect non-dividing memory CD4+ T-cells, macrophages and microglial cells in vivo (Freed and Martin, 2001). Several viral and cellular determinants have been reported to have an impact on active nuclear transport of HIV-1, suggesting that there is some redundancy or that the virus has evolved to use several pathways for nuclear import (Fassati, 2006). Interestingly, CA has also been implicated in HIV-1 nuclear import. The first evidence that CA influenced HIV-1 infection of non-dividing cells came from experiments using chimeric viruses where the Gag regions of HIV and MLV were swapped. If HIV-1 p17 MA was replaced with MLV matrix, the resulting virus could still infect non-dividing cells, however if p24 CA was replaced with MLV p30 CA, then the resulting virus could no longer infect non-dividing cells and in fact acquired an MLV phenotype, suggesting that CA was a dominant determinant (Yamashita and Emerman, 2004).

One reasonable interpretation of these results is that uncoating is different in the HIV/MLV chimera, and that MLV CA dictates slower uncoating, formation of a bulkier RTC/PIC, which is not able to go across NPCs (Fassati and Goff, 1999; Yamashita and Emerman, 2004). In support of this interpretation there are data showing that certain mutant HIV-1 CA do indeed uncoat more slowly and incompletely and are impaired in nuclear import (Dismuke and Aiken, 2006). However HIV-1 CA may also have a positive role in nuclear import. Several genome-wide siRNA screenings for host cell factors implicated in HIV-1 infection identified nucleoporin (Nup) 358 (also called RanBP2), Nup153 and the nuclear transport factor Transportin 3 (Tnpo3) (Brass et al., 2008; Konig et al., 2008). Importantly, it was shown that a specific mutation in CA at position N74 determined the ability of HIV-1 to use Nup153, Tnpo3 and, to some extent, also Nup358 (Lee et al., 2010). This particular mutation is located at the interface between NTD and CTD interactions forming the CA hexameric ring (Pornillos et al., 2009) (Fig. 1C) and was selected by passaging HIV-1 in the presence of a dominant negative truncated form of the splicing factor CPSF6, which prevents viral nuclear import when overexpressed in certain cell types (Lee et al., 2010). Subsequent work confirmed that Nup153 was important for HIV-1 infection and that the viral determinant mapped to CA, however knock down of Nup153 in target cells reduced the formation of 2LTR circular DNA (a hallmark of nuclear entry of the viral genome) only partially but had a more pronounced effect on integration (Matreyek and Engelman, 2011).

Tnpo3 is a member of the importin β superfamily of proteins that act as nuclear import or export receptors (or both) depending on whether they bind or release the cargo in the presence of RanGTP. Nuclear import receptors bind their cargos in the cytoplasm and release them in the nucleus upon binding to RanGTP, whereas nuclear export receptors bind their cargos in the nucleus in complex with RanGTP and dissociate from them in the cytoplasm upon hydrolysis of RanGTP (Gorlich and Kutay, 1999).

Initially it was thought that Tnpo3 stimulated HIV-1 nuclear import by binding to IN (Christ et al., 2008; Logue et al., 2011), however it later became apparent that the main viral determinant

for Tnpo3 is CA and that Tnpo3 stimulates HIV-1 nuclear import weakly. In fact Tnpo3 mainly affects post-nuclear entry steps (De Iaco and Luban, 2011; Krishnan et al., 2010; Valle-Casuso et al., 2012; Zhou et al., 2011) and can bind directly to the viral core (Valle-Casuso et al., 2012; Zhou et al., 2011), more efficiently when incubated in the presence of RanGTP (Zhou et al., 2011), which suggests that Tnpo3 can act as a nuclear export receptor for CA. Interestingly, there is evidence accumulating that Tnpo3 is also a nuclear import factor for CPSF6, an SR-rich splicing protein (Kewalramani, personal communication), and that CPSF6 binds directly to CA within a relatively large pocket (Lee et al., 2012; Price et al., 2012). This leads to several possible models to explain why Tnpo3 is required for HIV-1 infection (Fig. 3). For example, lack of Tnpo3 could cause abnormal CPSF6 accumulation in the cytoplasm, where it would bind HIV-1 CA, perturbing the viral life cycle, not too dissimilar from a restriction factor (Kewalramani, personal communication). Alternatively, CPSF6 by binding to CA could promote some event either before or after nuclear import to facilitate HIV-1 infection (Price et al., 2012). Another model proposes that, notwithstanding CPSF6 binding to CA, Tnpo3 is necessary to complete the removal of any remaining CA from the viral PIC inside the nucleus to allow efficient integration (Zhou et al., 2011) (Fig. 3). This third model will be elaborated further.

Curiously, other lentiviruses such as equine infectious anemia virus (EIAV) and feline immunodeficiency virus (FIV) do not depend on Nup153 and Tnpo3 for infection whereas SIVmac and HIV-2ROD do (Krishnan et al., 2010; Matreyek and Engelman, 2011), confirming that different lentiviruses can use different host components for nuclear entry and post-nuclear entry events, as previously suggested (Zaitseva et al., 2009).

Nup358 was also shown to be important for HIV-1 infection in several studies (Konig et al., 2008; Lee et al., 2010; Ocwieja et al., 2011; Schaller et al., 2011; Zhang et al., 2010). It is a 358 kDa protein that forms long filaments protruding in the cytoplasmic side of NPCs and it contains a Cyp-domain at its C-terminus (Wu et al., 1995). Importantly, HIV-1 CA binds the Cyp-domain of Nup358 directly and depletion of Nup358 results in a defect of HIV-1 infectivity that maps primarily at the step of nuclear import (Schaller et al., 2011). Therefore Nup358 may help HIV-1 dock at the nuclear pore. The flexible and filamentous nature of this nucleoporin would seem well suited to "embrace" a large nucleoprotein complex and retain it in close proximity of the NPC, where an appropriate reorganization can take place in preparation for the actual transport event. This mechanism implies that at least some CA remains associated with the HIV-1 RTC/PIC until NPC docking. Nup358 might also promote further uncoating of the viral core or even initiate uncoating of those cores that did not disassemble in the cytoplasm (Schaller et al., 2011) (Fig. 3). The idea that a partially disassembled core reaches the NPC filaments may also explain how certain tRNA species, which are incorporated into the HIV-1 particle during budding and have been shown to promote nuclear import of the RTC/PIC, are retained within the viral complex and then exposed to the import machinery at the right time (Zaitseva et al., 2006).

Perhaps not surprisingly, use of Nup358 is influenced by the ability of CA to bind CypA (Schaller et al., 2011), which could be due to the fact that the same region of CA is involved in binding to both CypA and Nup358 and/or that CypA may affect both the kinetics and the degree of uncoating, and hence ultimately the need to exploit Nup358 for infection. Indeed it is reasonable to assume that RTCs that completed uncoating in the cytoplasm and have very little or no CA associated may not need Nup358 or at least may not bind to it at the cytoplasmic side of the NPC.

Consistent with this possibility, the N74D CA mutant is more sensitive to CsA and it cannot infect macrophages efficiently (Ambrose et al., 2012). This block to infection was mapped prior to reverse transcription, suggesting that the N74D mutant uncoats too
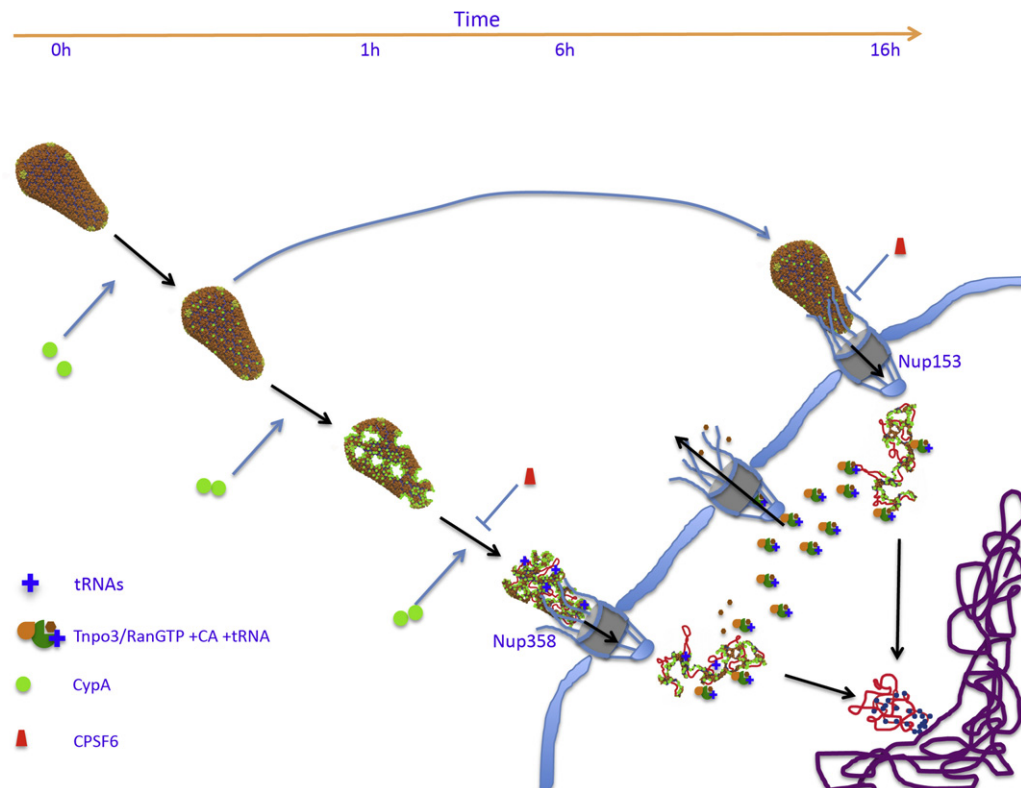
Fig. 3. Models for the multiple roles of CA in the early steps of HIV-1 infection. Shortly after entry of the HIV-1 core in the cell cytoplasm, CypA binds to CA. At low density CypA stabilizes the hexameric CA lattice, promoting the early steps of reverse transcription. However over time more CypA binds to the viral core until the density in certain regions is high (ratio CA:CypA close to 1:1) and the CA subunits in the lattice are dissociated, generating local collapse of the core structure. The process of CA disassembly carries on during reverse transcription, until the RTC binds to Nup358. Once the RTC/PIC docks to nuclear pores, further uncoating and other structural rearrangements occur and the complex is transported into the nucleus by several factors (importins, tRNAs) via an interaction with Nup153. Once inside the nucleus, residual CA, tRNAs and possibly other components are dissociated by Tnpo3 in complex with RanGTP. The stripped complex is now able to bind different cellular factors that are important for efficient and targeted integration into genomic DNA. The viral core can also bind to the NPC and disassemble locally, possibly using Nup358 as an uncoating factor, which replaces CypA. Cytoplasmic CPSF6 is shown in this model to block uncoating and thus prevent nuclear import and/or integration. An alternative model proposes that CPSF6 can facilitate RTC/PIC nuclear import by binding to CA. Mutant viruses or other lentiviruses that completely dissociate CA at an early stage must presumably use a different pathway that does not require Nup358, Nup153 and Tnpo3.

quickly and too much, making it unable to interact with Nup358, Nup153 and Tnpo3.

5. CA and integration

There is growing evidence indicating that CA plays a role in post-nuclear entry events, including integration. Certain CA mutations such as N54A/N57A and Q63A/Q67A cannot infect efficiently cells arrested in the cell cycle, however a careful analysis of the step impaired by these mutations showed that the block occurs after nuclear entry (Qi et al., 2008; Yamashita and Emerman, 2009; Yamashita et al., 2007). These mutations are located at the interface of the NTD–NTD intermolecular CA interactions that form the hexameric ring and may therefore change the stability of the hexamer itself (Pornillos et al., 2009) (Fig. 1C). Because these mutants show defects in uncoating, it was proposed that "total uncoating is the rate limiting step for efficient infection of nondividing cells" (Yamashita et al., 2007).

A functional link between HIV-1 CA and integration has also been described using a chemical genetic approach, which revealed that the small molecule Coumermycin-A1 impaired integration by targeting HIV-1 CA (Vozzolo et al., 2010). Interestingly, the A105S CA mutation makes the virus insensitive to this block (Vozzolo et al., 2010) and at the same time makes it independent of Tnpo3 for infection, suggesting that Coumermycin-A1 and lack of Tnp3 perturb the same pathway. The presence of CA associated with the HIV-1 PIC inside the nucleus can also be inferred from genetic studies in which

the restriction factors Fv-1 and members of the TRIM protein family were fused to CypA. The resulting fusion proteins maintained their specific ability to bind CA yet restricted HIV-1 at a post-nuclear entry step (Schaller et al., 2007; Yap et al., 2006). Furthermore, certain CA mutations, including N74D, show a different integration pattern in host chromosomes compared to wild type virus, which can also be observed upon depletion of Tnpo3 and Nup358 (Ocwieja et al., 2011; Schaller et al., 2011). Lastly, small amounts of CA could be detected in the nuclei of acutely infected cells by immunofluorescence and cell fractionation approaches (Zhou et al., 2011).

All the above evidence argues in favor of a link between CA, integration efficiency and integration targeting, therefore it is conceivable that "total uncoating" can happen in the nuclei, particularly in cells arrested in the cell cycle where normal uncoating might be less efficient or slower. In agreement with this possibility, Tnpo3 was shown to promote uncoating in the nucleus of infected cells by acting as an export factor for CA and certain tRNAs species (Zhou et al., 2011). Once the viral complex has translocated across NPCs, Tnpo3 would act as an export factor for residual CA and other elements bound to the complex, and by displacing these elements it would favor binding of other host factors present in the nucleus critical for efficient and targeted integration. This would occur only in the presence of RanGTP inside the nuclei. Hence Tnpo3 would orchestrate a relay race where different host factors bind to and are released from the HIV-1 RTC/PIC in a regulated and timely manner until its final destination is reached. The RanGDP/RanGTP

gradient across the nuclear envelope would ensure that host factors are interchanged in the right compartment (Fig. 3). On the other hand, mutant viruses that uncoat too fast or too completely, or cannot bind certain host factors, would not follow this pathway and therefore would become independent of Nup358, Nup153 and Tnpo3. Their RTC/PICs would presumably be in a different conformation, unable to bind the required host factors and hence would integrate into different genomic regions.

6. Small compounds targeting CA

Given the multiple and important roles of CA in the early steps of HIV-1 infection, significant efforts are being made to develop small compounds that can interfere with CA function. In fact several compounds and peptides binding to CA have been shown to perturb viral particle assembly and maturation (Jin et al., 2010; Lemke et al., 2012; Sticht et al., 2005; Tang et al., 2003). Remarkably, compounds that bind to the same pocket in CA but have a slightly different chemical structure can have different effects, such as inhibiting assembly or maturation, underlying the tight functional organiza-tion of the CA lattice (Lemke et al., 2012). In addition to compounds that block the late stages of the viral life cycle, more recently three compounds have been reported to inhibit early replication stages by targeting CA. Compound PF-74 was developed by Pfeizer start-ing from a high through put screening for inhibitors of HIV-1 and it binds to a preformed pocket in the CA NTD delimited by helices 3, 4, 5 and 7 (Blair et al., 2010). PF-74 affects primarily early reverse transcription, presumably by de-stabilizing the core prematurely through a loosening of the NTD–CTD intermolecular CA interac-tions or by preventing some host factor such as CPSF6 to bind the incoming core (Blair et al., 2010; Price et al., 2012; Shi et al., 2011). PF-74 also inhibits late stages of the HIV-1 life cycle by perturbing formation of the typical mature conical core (Blair et al., 2010). At the same time PF-74 was developed, Coumermycin-A1, an antibi-otic originally developed by Roche, was also shown to inhibit HIV-1 integration by targeting CA (Vozzolo et al., 2010) and molecular docking indicated that this compound binds to an extended pocket in the NTD CA, which includes the PF-74 binding pocket (Zhou et al., in preparation). Therefore, similarly to other compounds recently described (Lemke et al., 2012), PF-74 and Coumermycin-A1 bind to the same CA region but appear to have different effects. Inter-estingly, the antiviral activity of both PF-74 and Coumermycin-A1 is positively modulated by CypA, which reinforces the view that these drugs impact on core uncoating (Shi et al., 2011; Vozzolo et al., 2010). Another series of small compounds binding to a dif-ferent pocket of the NTD CA has been described, also impairing mainly reverse transcription (Kortagere et al., 2012). These results show that CA is indeed a promising target for drug development and suggest the possibility that even small differences in the chem-ical structure of the compounds might lead to alternative ways to block HIV-1 infection. One important question concerns the genetic barrier to these compounds and how difficult will be for viruses to escape the effects of such drugs. Nonetheless, it is an area of much interest and promise.

7. Concluding remarks

In conclusion, it is becoming increasingly apparent that CA has multiple roles during the early stages of HIV-1 infection. CA links into intrinsic and innate immune recognition, nuclear import and integration and may bind to host cell factors that directly or indirectly influence such events. Using a combination of genetic, biochemical and imaging approaches, new aspects of HIV-1 biology are being discovered, which will illuminate important cellular path-ways and will lead to the development of new small molecules with

therapeutic potential. Many questions remain, for example if there are additional roles for Nup153, Nup358 and Tnpo3 in HIV-1 infec-tion, if additional host cell factors or restriction factors that bind CA are involved in the early steps of HIV-1 infection, how PF-74, Coumermycin A1 and other small compounds work, what is their relationship with CypA, why the phenotype of several CA mutants is cell-type dependent and what is the impact of these factors on HIV-1 transmission and the natural history of HIV-1 infection. We are still in the early days of the early steps of HIV-1 infection!

Acknowledgements

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