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Determinants of the HIV-1 core assembly pathway

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ABSTRACT

Based on structural information, we have analyzed the mechanism of mature HIV-1 core assembly and the contributions of structural elements to the assembly process. Through the use of several *in vitro* assembly assay systems, we have examined details of how capsid (CA) protein helix 1, ß-hairpin and cyclophilin loop elements impact assembly-dependent protein interactions, and we present evidence for a contribution of CA helix 6 to the mature assembly-competent conformation of CA. Additional experiments with mixtures of proteins in assembly reactions provide novel analyses of the mature core assembly mechanism. Our results support a model in which initial assembly products serve as scaffolds for further assembly by converting incoming subunits to assembly proficient conformations, while mutant subunits increase the probability of assembly termination events.

Introduction

The assembly of HIV-1 virus particles is controlled by the 55 kDa polyprotein precursor Gag (PrGag) protein, which is the key structural protein of HIV-1 (Wills and Craven, 1991; Mervis et al., 1988; Wang et al., 1998; Spearman et al., 1994; Reicin et al., 1996; Hermida-Matsumoto and Resh, 1999; Freed, 1998; Ono et al., 2000; Huseby et al., 2005). PrGag is composed of four major domains: matrix (MA), capsid (CA), nucleocapsid (NC), and p6, as well as two small spacer peptides, SP1 (between CA and NC) and SP2 (between NC and p6) (Erickson-Viitanen et al., 1989; Kräusslich et al., 1995; Swanstrom and Wills, 1997; Wiegers et al., 1998; Borsetti et al., 1998; Burniston et al., 1999). MA is the component of PrGag that is responsible for its binding to the plasma membrane (Zhou et al., 1994; Dalton et al., 2007), which helps trigger immature virus particle release (Ono et al., 2000; Li et al., 2007). During or just after immature particle release, proteolytic processing of PrGag results in a conformational change within CA that induces viral morphogenesis. As visualized by electron microscopy (EM), electron dense conical or cylindrical cores composed of about 1000–1500 CA proteins are evident in mature virus particles (Briggs et al., 2003, 2006; Li et al., 2000). Also present within mature cores are NC–viral RNA complexes, and experimental data support a model in which NC binding to RNA helps nucleate core assembly, perhaps by fostering CA dimerization (Dorfman et al., 1993; Gorelick et al., 1993; Berkowitz et al., 1995; Zhang and Barklis, 1997; Zhang et al., 1998; Dawson and Yu, 1998; Cimarelli et al., 2000; Alfadhli et al., 2005; de Marco et al., 2010). Via microscopy analysis of mammalian cells transfected with HIV constructs, it recently has been validated that the viral RNA (vRNA) plays a scaffolding role in HIV-1 assembly, and that complexes of NC–vRNA located at cellular plasma membranes are highly favored sites for assembly (Jouvenet et al., 2009).

Mature HIV-1 CA possesses two independently folded domains; an N-terminal domain (NTD) that is important for formation of CA hexamers, and a C-terminal domain (CTD), that contributes to hexamer formation and also fosters CA dimerization that is essential for particle assembly (Gitti et al., 1996; Momany et al., 1996; Gamble et al., 1997; Berthet-Colominas et al., 1999; Worthylake et al., 1999; Ganser-Pornillos et al., 2007; Pornillos et al., 2009). The CA NTD is composed of seven alpha helices, a cyclophilin (CypA) binding loop between helices 4 and 5, and an N-terminal ß-hairpin loop composed of the first thirteen residues of CA. The interface formed by helices 1 and 2 makes NTD–NTD contacts that are essential for the assembly and stabilization of CA hexamers both *in vivo* and *in vitro*, whereas truncation of the CypA loop fosters the *in vitro* assembly of CA cores (von Schwedler et al., 2003; Douglas et al., 2004; Ganser-Pornillos et al., 2007; Barklis et al., 2009). Other studies have demonstrated that the N-terminal ß-hairpin loop becomes stabilized after processing by a salt bridge formed between residues 1 (proline) and 51 (aspartic acid) and that the presence of the ß-hairpin correlates with mature core assembly (Gamble et al., 1996; Gitti et al., 1996; Gross et al.,

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1998; von Schwedler et al., 1998; Tang et al., 2002; Abdurahman et al., 2007; Monroe et al., 2010). The CA CTD is smaller than the NTD and is composed of a short 3_{10} helix followed by an extended strand and four α-helices (Momany et al., 1996; Gamble et al., 1997; Berthet-Colominas et al., 1999; Worthylake et al., 1999; Alcaraz et al., 2007; Wong et al., 2008; Pornillos et al., 2009; Byeon et al., 2009). The CTD appears to have the capacity to dimerize in several ways (Gamble et al., 1997; Worthylake et al., 1999; Ternois et al., 2005; Ivanov et al., 2007; Byeon et al., 2009), and the dimerization interface depends on residues W184 and M185 (Gamble et al., 1997; Alcaraz et al., 2008; Byeon et al., 2009; Yu et al., 2009). Recent evidence suggests that an interface formed by the CTD dimers of three neighboring CA hexamers might be involved in organizing intermolecular contacts of mature HIV-1 cores (Byeon et al., 2009). In addition to the formation of dimer and hexamer subunits, retroviral CA proteins have the capacity to assemble pentamers, which allow capsid lattices to make closed core structures similar to those of fullerene cones (Ganser et al., 1999 and Li et al., 2000; Pornillos et al., 2011). Based on this type of structure, HIV-1 cores are modeled to be composed of hexameric and pentameric subunits, with seven pentamers located at one end of the core, and five located at the narrow core end (Ganser et al., 1999; Li et al., 2000; Pornillos et al., 2011).

A model for HIV-1 core assembly suggests that three steps may be differentiated: a slow nucleation step, in which the nucleation species is still unknown; a growth step that involves a fast polymerization of the CA protein; and a termination step, which might involve the capping of growing tubes (Barklis et al., 2009). Despite the current structural information of CA hexameric and pentameric forms, it is still not certain how and whether dimers, hexamers and/or pentamers contribute to the nucleation, growth or termination steps during core assembly, and consequently the mechanisms by which HIV-1 CA proteins assemble to form mature cores *in vivo* and *in vitro* are still unknown. One puzzle is how postulated nucleation complexes might serve as templates for rapid polymerization. Also unknown are the factors that dictate sphere versus core or tube assembly, although theoretical studies imply that the monomer orientations of CTDs and NTDs may regulate the curvature of CA lattices (Nguyen et al., 2006; Levandovsky and Zandi, 2009; Krishna et al., 2010).

Several reports have shown that CA cores assembled *in vitro* resemble those observed *in vivo* (Li et al., 2000; Briggs et al., 2003; Ganser-Pornillos et al., 2007; Byeon et al., 2009) affording investigators an avenue to understanding the principles of mature core assembly. *In vitro* assembly reactions using purified HIV-1 CA proteins result in the formation of long tubes, although cones and spheres can be also observed. In this study, we have examined factors that regulate CA assembly using turbidity assays, fluorescence microscopy (FM) and electron microscopy (EM). We have focused our analysis on amino acid residues located on CA NTD helices 1 and 6, and the ß-hairpin and cyclophilin loops, to determine whether perturbations of these residues result in assembly defects that help elucidate the mechanisms of HIV-1 mature core assembly. Using this strategy, we have characterized CA variants that show disparate assembly properties. Replacements of amino acids located at the N- but not C-terminus of helix 1 (I15, R18), and in the hairpin loop (Δ1–16, H12) impaired mature core assembly. Interestingly, amino acid replacements of residues situated in the upper face region of CA contained by NTD helix 6 (L111, W117), which is adjacent to the ß-hairpin and Cyp-A loops, also impacted core formation, indicating that NTD helix 6 is also a key element that regulates CA assembly.

We further determined that one of our helix 1 mutant proteins was capable of acting as a dominant negative with regard to CA tube assembly, suggesting that the equilibrium between tube and sphere formation can be easily skewed towards the termination of core polymerization. Finally, EM experiments using capsid plus nucleocapsid (CANC) proteins and RNA to foster CA oligomerization demonstrate that CA proteins appear to be triggered to switch conformations as they oligomerize, and that assembled proteins facilitate the conversion of new subunits to an assembly competent status. Mechanisms by which nucleation scaffolds thus regulate HIV-1 core assembly are discussed.

Results

Effects of CA mutations on assembly

To help elucidate the mechanism of mature HIV-1 core assembly, we have analyzed factors that regulate the *in vitro* assembly of capsid tubes from HIV-1 CA monomers. We examined the effects of mutations targeted in CA NTD helices 1 and 6, and the β-hairpin and cyclophilin loops. The CA residues on helix 1 targeted for a mutational analysis were isoleucine 15 (I15), proline 17 (P17), arginine 18 (R18), asparagine 21 (N21) and lysine 25 (K25) (Figs. 1A and B). I15 was mutated based on the notion that this residue might be involved in the correct positioning of the β-hairpin loop that is formed during HIV-1 core maturation (Fig. 1B), whereas residues R18, N21 and K25 possess side chains that point toward neighbor CA proteins within hexamers, potentially organizing CA hexameric lattices (Fig. 1A). We examined a ß-hairpin loop deletion protein (Δ1–16; Fig. 1) to extend previous observations (Gross et al., 1998), which suggested that deletion of the first 13 residues of CA was compatible with assembly. We also tested the effects of mutating one residue located within the hairpin loop (H12; Figs. 1C and D), one residue in the CypA loop (M96; Figs. 1C and D), and a deletion of the CypA loop itself. To complement these studies, other residues located in the upper face loop region of CA NTD were targeted. In particular, mutations at helix 6 residues L111 and W117 were generated. These were of interest because helix 6 is adjacent to the biologically important ß-hairpin and CypA loops, and the influence of helix 6 on assembly has as yet not been investigated in detail.

To monitor how mutations affected the kinetics of CA assembly we measured oligomerization of purified WT and mutant CA proteins spectrophotometrically (Lanman et al., 2002; Tang et al., 2003; Douglas et al., 2004; Sticht et al., 2005; del Alamo et al., 2005; Abdurahman et al., 2007; Barklis et al., 2009). The time-dependent formation of light-scattering CA assembly products was monitored for 60 min, and examples of turbidity assay plots are provided in Figs. 2A and B. In Figs. 2C and D maximum absorbance readings from multiple experiments are graphed for all the mutant proteins. As illustrated in Fig. 2A, the P17A mutant CA, as well as the conservative K25R mutant assembled efficiently, whereas the mutant proteins I15D, R18L and, N21L assembled considerably less well than the WT counterpart. As shown in Fig. 2B, one of the helix 6 mutants, L111W, showed higher turbidity values than WT, while the Δ87–97, H12A, and M96A mutants gave roughly WT results. Relative to WT, several helix 1 mutant proteins (I15D, R18H, R18L, R18E, N21L; Figs. 2A and C) gave low turbidity values, indicative of the importance of helix 1 in making protein contacts critical to mature core assembly. We also observed reduced signals from the Δ1–16 ß-hairpin mutant (Figs. 2B and D), consistent with assignment of the ß-hairpin as a regulator of core morphology. Additionally, several helix 6 variants (L111A, L111F, W117A; Figs. 2B and D) demonstrated low to minimal turbidity signals. This was unexpected, because helix 6 previously has not been earmarked as a determinant of core assembly.

Because turbidity assays cannot distinguish between properly assembled tubes and non-specific protein aggregation, we also monitored CA assembly by fluorescence microscopy (FM), using a method that we recently developed for this purpose (Barklis et al., 2009). Briefly, assembly products are adhered to glass coverslips and detected by immunofluorescence such that tubes appear as bright lines against a black background. As an example, in Figs. 3A–D, FM images of the WT, I15D, Δ1–16 and L111W assembly products are shown. Assembled CA tubes were evident as fluorescent (white) lines, particularly with the WT (Fig. 3A) and L111W (Fig. 3D) samples. Such products were rarely seen in the I15D and Δ1–16 proteins (Figs. 3B