Cryo-electron microscopy of tubular arrays of HIV-1 Gag resolves structures essential for immature virus assembly

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The assembly of HIV-1 is mediated by oligomerization of the major structural polypeptide, Gag, into a hexameric protein lattice at the plasma membrane of the infected cell. This leads to budding and release of progeny immature virus particles. Subsequent proteolytic cleavage of Gag triggers rearrangement of the particles to form mature infectious virions. Obtaining a structural model of the assembled lattice of Gag within immature virus particles is necessary to understand the interactions that mediate assembly of HIV-1 particles in the infected cell, and to describe the substrate that is subsequently cleaved by the viral protease. An 8-Å resolution structure of an immature virus-like tubular array assembled from a Gag-derived protein of the related retrovirus Mason-Pfizer monkey virus (M-PMV) has previously been reported, and a model for the arrangement of the HIV-1 capsid (CA) domains has been generated based on homology to this structure. Here we have assembled tubular arrays of a HIV-1 Gag-derived protein with an immature-like arrangement of the C-terminal CA domains and have solved their structure by using hybrid cryo-EM and tomography analysis. The structure reveals the arrangement of the C-terminal domain of CA within an immature-like HIV-1 Gag lattice, and provides, to our knowledge, the first high-resolution view of the region immediately downstream of CA, which is essential for assembly, and is significantly different from the respective region in M-PMV. Our results reveal a hollow column of density for this region in HIV-1 that is compatible with the presence of a six-helix bundle at this position.

cryo-electron tomography | helical reconstruction | SP1 | electron cryomicroscopy

The major structural component of HIV-1 is the 55-kDa Gag polypeptide. Gag proteins from all retroviruses contain an N-terminal membrane-binding matrix (MA) domain; two central domains that together comprise capsid (CA); and an RNA-binding nucleocapsid (NC) domain located toward the C terminus of Gag. Between, and downstream of these domains are regions that differ between retroviruses. In HIV, a spacer peptide, SP1, is found between CA and NC, whereas downstream of NC are a second spacer peptide, SP2, and the p6 domain (1).

Assembly of an infectious HIV-1 particle proceeds in two stages (2). In the first stage, Gag oligomerizes into a curved lattice on the cytoplasmic face of the plasma membrane, leading to outward protrusion of the membrane and subsequent membrane scission to release an immature virus particle. Within the immature virus particle, Gag is arranged in a radial manner with the N-terminal MA domain on the inner surface of the membrane, and the C terminus of Gag pointing toward the center of the particle. In the second stage of assembly, the viral protease becomes activated and cleaves Gag at five positions, releasing the component domains, leading to a structural rearrangement within the virus particle. MA remains bound to

the viral membrane, NC and the associated viral genome condense in the center of the particle, and CA assembles to form a conical core around the viral genome. This proteolytic maturation is required for the particle to become infectious and is a target of antiretroviral drugs (3). The immature virus particle therefore represents a key intermediate in HIV-1 assembly. Obtaining detailed structural information on the arrangement of Gag within immature HIV-1 is essential to reveal the protein interfaces that mediate assembly of the immature virus, and to reveal the structure of the substrate that must be cleaved by the viral protease to induce maturation to the infectious form.

There has been an extensive effort to apply structural biology methods to understand the structures and interactions that mediate assembly of HIV-1, and the structural changes associated with maturation (4, 5). High-resolution structural data exist for

Significance

HIV-1 undergoes a two-step assembly process. First, an immature noninfectious particle is assembled, which leaves the infected cell. Second, the structural protein, Gag, is cleaved in the virus by the viral protease, and this leads to formation of the infectious virus. The immature virus particle therefore represents the key intermediate in HIV-1 assembly. There is currently no high-resolution information available on the arrangement of Gag within immature HIV-1. We have assembled part of HIV-1 Gag in vitro to form immature virus-like tubular protein arrays, and have solved a subnanometer-resolution structure of these arrays by using cryo-EM and tomography. This structure reveals interactions of the C-terminal capsid domain of Gag that are critical for HIV-1 assembly.

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Data deposition: The X-ray structures and the fit have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4COC, 4COP, and 4D1K) and the cryoEM structure has been deposited in the Electron Microscopy Data Bank (EMDB number EMD-2638).

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all the folded domains of HIV-1 Gag in their isolated forms. Recently, a series of breakthrough studies have revolutionized our understanding of how the CA domains self-assemble to form the mature capsid core of the virus (6–8), and a pseudoatomic model for the assembled mature core is now available (9). In contrast, much less structural information is available on the immature virus. A structure of the HIV-1 Gag lattice within immature-like particles assembled *in vitro* from a Gag-derived protein has been resolved to a resolution of ~ 17 Å by using subtomogram averaging (10). At this resolution, the approximate positions of the protein domains within the lattice can be defined, but their orientation is not clearly determined. We recently solved the structure of immature-like tubular assemblies of a Gag-derived protein (Δ ProCANC) from another retrovirus, Mason–Pfizer monkey virus (M-PMV), to a resolution of ~ 8 Å (11). At this resolution, the arrangement of the CA domains could be clearly determined, revealing the regions of CA that interact within the assembled immature M-PMV Gag lattice. Based on the expected structural homology between the HIV-1 and M-PMV CA domains, a model for the arrangement of the CA domains in immature HIV-1 was generated and compared with the CA arrangement in mature HIV-1 cores. Strikingly, the interfaces that stabilize the immature and the mature CA lattices are almost completely distinct in this model.

The SP1 peptide plays a crucial role in HIV-1 assembly and maturation (2). A stretch of ~ 25 residues downstream of the folded C-terminal domain of CA comprising the C-terminal residues of CA and SP1 is known to be essential for immature HIV-1 assembly (12, 13). This stretch encompasses the CA–SP1 proteolytic cleavage site, mutation of which abolishes formation of mature cores and infectious viruses (14). The bevirimat class of HIV-1 assembly inhibitors blocks HIV-1 replication by specifically targeting processing at this site (15). The structure of this important region of Gag has not yet been resolved with high resolution, however. Early mutational studies and molecular modeling led to the proposal that this region forms an extended α -helix (13), and this was supported by subsequent NMR studies of the isolated sequence (16). The presence of thick columns of density linking the CA and NC layers in low-resolution EM structures is consistent with an extended six-helix bundle forming in this region (10, 13, 17). M-PMV, which contains no spacer peptide between CA and NC, diverges structurally from HIV-1 in this region (18), and, instead of the thick density columns, six splayed densities were observed in the M-PMV Δ ProCANC tubes (11).

The available structural data therefore do not answer several critical questions: Most importantly, (i) does the arrangement of the CA domains in immature HIV correspond to the model generated based on structural homology from the M-PMV-derived tube structure; and (ii) what is the structure of the region immediately downstream of CA that is critical for immature assembly and the regulation of maturation?

The key step in obtaining detailed structural data for the immature M-PMV lattice was to generate immature-like tubular arrays of a Gag construct. The helical symmetry of the M-PMV Δ ProCANC tubes allowed application of a hybrid cryo-electron tomography (ET) and cryo-EM image processing approach for structure determination (11). HIV-1 Gag derivatives that assemble tubular arrays adopt a mature-like arrangement of CA (19, 20), and have indeed been important tools in determining the structure of the mature CA core (9, 21). In contrast, HIV-1 Gag derivatives known to assemble into immature-like arrays form approximately spherical structures (22, 23) that are unsuitable for application of high-resolution helical reconstruction methods.

Here we identify an HIV Gag-derived protein competent to assemble partially immature-like tubular arrays, and determine their structure to a resolution of 9.4 Å. The structure reveals the

immature-virus-like arrangement of the C-terminal domain of HIV CA (CA-CTD) and the CA–SP1 linker region.

Results and Discussion

Immature-Like Tubular Arrays. We previously assembled immature-like tubular arrays of M-PMV Gag from a protein comprising CA, NC, and the intervening residues, but lacking the N-terminal proline residue of CA (Δ ProCANC) (11). Similar HIV-1 Gag-derived proteins assemble narrow tubular arrays (Fig. 1A) (19, 20, 24), which have a mature-like arrangement of CA. Prior studies had indicated that certain HIV-1 Gag-derived proteins can assemble tubular or spherical particles depending on the conditions (22) and that certain mutations in the CA–SP1 region converted the assembly from spherical to tubular or aberrant sheet-like structures (12, 25). Furthermore, mutations in the region targeted by the capsid assembly inhibitor CAI do not alter assembly of immature particles *in vitro* or in tissue culture, whereas they completely disrupt assembly of the mature-like lattice (26). We therefore purified CANC variants and other Gag-derived proteins with these mutations, and tested them for assembly of wide tubular particles. We found that CANC proteins with the mutation Y169L or Y169S yielded tubes with a wider diameter upon negative stain EM, whereas all other

Fig. 1. Arrangement of the Gag lattice is immature-like in the HIV-1 CANC Y169L tubes. (A) WT HIV-1 CANC protein was assembled along with DNA. On a slice through a tomogram of the assembled tubes, thin mature-like tubes were observed. (B) A single amino acid mutation Y169S/L converts thin mature-like tubes to thick immature-like tubes (Fig. S1). (C) Comparison of radial density profiles of CANC Y169L tubes with immature HIV-1 virus particles and mature-like WT CANC tubes. (D) A schematic representation of the HIV-1 Gag polyprotein aligned with the radial density profile shown in C (green). Positions of the NC, SP1, CA-CTD, CA-NTD, and MA domains along with the viral membrane are indicated.

Fig. 3. Arrangement of CA domains in HIV-1 CANY169S/L tubes. (A) The arrangement of the CA lattice on the HIV-1 CANY169S/L tubes viewed from outside the surface of the tube. The CA-NTD domains are colored blue and the CA-CTD domains are colored orange. A gray hexagon is superimposed on the image to show the pseudohexameric asymmetric unit on the surface of the tubes. (B) Arrangement of the CA-NTD lattice. (C) Arrangement of the CA-CTD lattice. Fig. S5 A and B show CA-NTD and CA-CTD symmetry axes. (D) The CA lattice viewed from the side of the tube. A gap is observed between the CA-NTD and CA-CTD layers. (E) Additional densities are observed in the final reconstruction, outside the fitted first seven α -helices of CA, which are consistent with the folded N-terminal β -hairpin. Isosurface threshold is 1.0σ away from the mean. (F) The CA-CTD and CA-NTD layers are connected by the 7–8 linker (arrowheads), which is flexible and forms three different arrangements (Fig. S6). Isosurface threshold is 0.75σ away from the mean.

Arrangement of the CA Domains. Unexpectedly, the CA-NTDs in the HIV-1 CANY169S/L tubes are not assembled to form a hexameric lattice, as had been observed in M-PMV Δ ProCANC tubes and in previous studies on immature HIV-1 Gag assemblies (10, 17), but instead pack together to form a lattice that is “p2” in crystallographic terms, containing four twofold positions (Figs. 2E and 3B and Fig. S5 A and C). Although a lattice with this symmetry can form a tubular array, it is difficult to adapt it to form spherical structures as would be necessary in the virus. Within the lattice, clear additional densities outside the positions of the fitted first seven CA α -helices are observed, corresponding to the N-terminal β -hairpin of the CA-NTD (Fig. 3E). This feature cannot be present in the immature virus, because the β -hairpin only forms upon proteolytic cleavage of the N terminus of CA from the preceding MA domain. The β -hairpin appears to mediate interactions between CA-NTDs in the HIV-1 CANY169S/L tubes. Taken together,

these observations suggest that the arrangement of the CA-NTD within the HIV-1 CANY169S/L tubes is unlikely to represent the arrangement of the CA-NTD within the immature virus and that it is most probably specific to the *in vitro* assembly of this mutant protein. We have therefore not interpreted it further. However, we note the remarkable capacity of the CA-NTD to oligomerize in at least three different ways to form curved lattices—first as seen in the mature HIV-1 capsid, second as seen in the M-PMV Δ ProCANC tubes, and third as seen in the HIV-1 CANY169S/L tubes described here.

The CA-CTD monomers are well resolved (Figs. 2F and 3C and Fig. S2 E and F). They form an approximately hexameric lattice, “p6” in crystallographic terms (Fig. 3C and Fig. S5 B and D). The lattice spacing derived from the helical parameters was 72.5 Å at the CA-CTD:SP1 interface, corresponding to 81 Å at the CA-NTD. This is in good agreement with the interhexameric distance of ~ 80 Å at the CA-NTD measured in the immature HIV-1 Gag lattice by previous cryo-EM studies (17, 31), and significantly smaller than the ~ 95 Å interhexameric spacing observed in the mature lattice (6). In the structure, there is a space between the CA-NTD and CA-CTD domains (Fig. 3D). The space between the domains is larger than that previously observed in the M-PMV Δ ProCANC tubes. No direct protein–protein contacts were observed between the two domains, but the polypeptide chain linking the domains (the 7–8 linker) is resolved (Fig. 3F and Fig. S3 C and D). Because of the mismatch between the p2 symmetry of the CA-NTD and p6 symmetry of the CA-CTD lattices, there are three different relative positions of the CA-CTD and CA-NTD. All these positions differ from the relative positions of the CA-CTD and CA-NTD in the M-PMV Δ ProCANC tubes and in the mature HIV-1 CA lattice (Fig. S6). To link the domains, there are therefore three different orientations of the 7–8 linker (Figs. S3 C and D and S6). Together, these data indicate that the arrangement of the CA-CTD within the immature-like lattice can be formed independent of the arrangement of the CA-NTD, and that the linker between them is highly flexible.

The CA-CTD layer is made of compact α -helical domains (Fig. 2F and Fig. S2 E and F). The overall arrangement of the CA-CTD monomers resembles that seen in the M-PMV Δ ProCANC tubes (11) and in one of the previously derived models of the immature HIV-1 CA-CTD lattice (10). The CA-CTD monomers in the HIV-1 CANY169S/L tubes show a kink in helix 9 of CA, a feature also observed in the X-ray atomic structure, but not observed in the M-PMV Δ ProCANC tubes. The dimer interface and the overall arrangement of the HIV-1 CA-CTD dimers around the hexameric axis are similar to that obtained for M-PMV (Fig. S3 E and F). In HIV-1 and M-PMV tubes, CA-CTD dimerization is mediated by a hydrophobic interface between helix 9 in each monomer (Figs. S3 E and F and S7). In HIV-1 CA, residues W184 and M185 are close to this interface, whereas, in M-PMV CA, residue Y180 (in the sequence VDYV) is close to this interface. The relative orientation of the CA-CTD monomers within a CA-CTD:CA-CTD dimer is similar to that in the M-PMV Δ ProCANC tubes (only 1° difference in the crossing angle of the two helix 9s), and to that in the crystal structure of CA Y169A (4° difference in crossing angles). In contrast, the relative orientation differs strongly from “mature-like” dimeric forms such as mature-like CA arrays (138° difference) or HIV-1 CA crystals (118° difference; Fig. S7). Despite the highly similar structures of the dimers, the sizes of the hexamers formed by these CA-CTD dimers are different: 72.5 Å for HIV-1 and 86 Å for M-PMV (measured at the CA–SP1 interface). To accommodate the smaller hexamer size, adjacent CA-CTDs around the sixfold axis are closer together in the HIV-1 CANY169S/L tubes (18.9 Å center to center) than in the M-PMV Δ ProCANC tubes (22.6 Å; Fig. S3 E and F). The interface between adjacent CA-CTDs around the sixfold is correspondingly more extensive in the HIV-1 CANY169S/L tubes

The hollow columns of density seen in the HIV-1 CMC Y169S/L tubes is in contrast to the six splayed densities that

Essentially all stages of the viral lifecycle have been proposed as potential targets for antiretroviral drugs (15), including assembly and cleavage of the immature virus. In the absence of structural data on the immature Gag lattice, the binding sites of assembly inhibitors relative to the protein–protein interfaces remain unknown. Indeed for inhibitors such as bevirimat, the binding site exists only in the assembled immature lattice. For the CA-CTD, the structure presented here provides the best structural model to date for the arrangement of the CA-CTD

and downstream regions in immature HIV-1, and, together with the M-PMV structure presented previously (11), provides a framework for understanding how inhibitor-binding sites relate to the positions of interfaces important in virus assembly. Mutagenesis analysis has shown the CA-CTD regions to be most critical in mediating virus assembly, and the interfaces they form during assembly include the most conserved residues across retroviruses. The observed similarity in CA-CTD:CA-CTD interactions between M-PMV and HIV makes it reasonable to assume that these interactions will be conserved across retroviruses.

Materials and Methods

Protein Purification and in Vitro Assembly. HIV-1 CA-CTD and CMC proteins were expressed and purified from *Escherichia coli* as described previously (22, 26). Assembly of purified proteins into tubes was induced as described previously (22). Immature HIV-1 particles (for radial-density profiles) were produced by transfection of 293T cells with the HIV-1 proviral plasmid pNL4-3 in the presence of 5 μ M amprenavir followed by purification as described previously (40). (SI Materials and Methods).

X-Ray Structure Determination of Y169S and Y169L CA-CTD Proteins. Crystallization, data collection, structure refinement, and model building of CA-CTD Y169S and CA-CTD Y169L proteins were performed as described previously (26) (SI Materials and Methods). PDB accession codes are 4COC for Y169L and 4COP for Y169S.

Cryo-EM and Image Processing. Cryo-EM sample preparation and data collection was performed as described previously (11). The helical parameters of each tube were extracted using cryo-ET and subtomogram averaging (10, 11, 41). Real-space helical reconstruction was conducted by using extracted helical parameters (42). The pseudohexameric asymmetric units from all reconstructed tubes were averaged to obtain a final reconstruction of the HIV-1 CMC Y169S/L tubes at 9.4 Å (SI Materials and Methods). The structure and fit are deposited under EMDB accession number EMD-2638, and PDB accession code 4D1K.

Atomic Structure Fitting and Analysis. Atomic structures of retroviral CA domains were fitted into the cryo-EM densities by using MDFF (30). Analysis of final obtained PDB files was done by using the UCSF Chimera package (43). (SI Materials and Methods).

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