High-resolution structures of HIV-1 Gag cleavage mutants determine structural switch for virus maturation
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The major structural component of HIV-1 is the 55-kDa polyprotein Gag. Gag oligomerization at the plasma membrane of infected cells directs the budding and release of enveloped immature virus particles. The Gag proteins of all retroviruses include three conserved domains: the MA (matrix) domain, which binds the plasma membrane; the CA (capsid) domain, which, upon cleavage, forms the mature viral capsid; and the NC (nucleocapsid) domain, which packages the viral RNA genome into a ribonucleoprotein (RNP) complex. HIV-1 Gag comprises, in addition, a short spacer peptide between CA and NC called SP1 and two further peptides, SP2 and p6, downstream of NC (Fig. 1A). Gag is radially arranged in the immature virus particle with the N-terminal MA domain at the membrane and the C-terminal end of the protein pointing toward the center of the virus particle. Between them, the CA domain forms a hexameric protein lattice arranged as an incomplete sphere containing irregularly shaped defects. The viral PR is expressed as an incomplete sphere containing irregularly shaped defects. The viral PR cleaves Gag at five positions, separating it into its component domains and peptides, and leading to rearrangement of the virus into its mature, infectious form (Fig. 1A). The viral PR is expressed as a part of the 160-kDa Gag-Pol polyprotein, which is produced by a ribosomal frameshift and incorporated into the immature lattice at ~5% abundance. In the mature virus particle, MA is believed to remain associated with the membrane; CA forms the cone-shaped mature capsid core; and NC, together with the viral RNA, forms a condensed RNP complex within the capsid (1–5).

The structures of both immature and mature HIV-1 particles have been extensively studied by cryo-electron tomography (cryo-ET) and subtomogram averaging (6–11), as well as by crystallography of purified protein components and multimers thereof (12–17). These studies have led to atomic models of the CA domain within the assembled Gag lattice of immature viruses (10, 17) and in mature viral capsids (11, 16) that reveal the networks of interactions that stabilize the CA domain lattice. In both cases, CA assembles a hexameric protein lattice, but the hexamer–hexamer spacing differs (18, 19) and the CA–CA interfaces that mediate interactions within the lattice are almost completely nonoverlapping (20). Some disassembly and reassembly of the interactions of the CA N-terminal domain (CA-NTD) and CA C-terminal domain (CA-CTD) are therefore required during maturation. Upon cleavage, the N-terminus of the CA-NTD folds down upon itself to form a beta-hairpin (21). Downstream of the CA-CTD, an alpha-helix that crosses the CA-SP1 boundary forms a hexameric bundle in the immature lattice (10, 17, 22) that is disassembled upon maturation, and the C-terminal part of CA is disordered in the mature lattice. Maturation is therefore associated with dramatic changes in CA structure, particularly at the termini of the protein, as well as changes in CA arrangement (reviewed in ref. 5) (Movie S1).

Significance
The main structural component of HIV-1 is the Gag polyprotein. During virus release, Gag is cleaved by the viral protease at five sites, triggering a major change in the structure and morphology of the virus. This transition, called maturation, is required to make an infectious virion. We used cryo-electron tomography to obtain high-resolution structures of Gag inside virus particles carrying mutations that block specific combinations of cleavage sites. Analysis of these structures suggests that different combinations of cleavages can destabilize a bundle of alpha-helices at the C terminus of CA. This destabilization, rather than formation of a beta-hairpin at the N terminus of CA as previously suggested, acts as the structural switch for maturation of the virus into its infectious form. Author contributions: H.-G.K. and J.A.G.B. designed research; S.M., A.T., and B.G. performed research; S.M., A.T., H.-G.K., and J.A.G.B. analyzed data; and S.M., A.T., B.M., H.-G.K., and J.A.G.B. wrote the paper.

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Despite the availability of detailed models for the immature and mature states, little is known about the intermediate states of HIV-1 maturation. The cleavages that release the domains of Gag proceed at very different rates, influenced by protein sequence and structure. In vitro, cleavage rates for different sites in the proteolytic cleavage cascade. (B) Schematic representation of Gag cleavage patterns in each of the cleavage mutants used. Mutated sites that cannot be cleaved are denoted with crossed out, dashed lines. Sites where cleavage occurs as in wild-type Gag are denoted by solid lines. Brackets under the schematics indicate the CA-containing products that remain unprocessed and their corresponding molecular mass. (C) SDS/PAGE analysis of virus preparations used for the structural analyses. Particles were purified from the supernatant of transfected HEK293T cells by ultracentrifugation through an iodixanol gradient. Samples were separated by SDS/PAGE (12.5% acrylamide, 30:1 acrylamide/bisacrylamide), and proteins were visualized by silver staining. Numbers to the left indicate the position of molecular mass standards (in kilodaltons). Purified recombinant Gag protein was used as a standard to estimate particle concentration. (D) Quantitative immunoblot analysis of MA-CA and MA-SP1 band intensities. Samples were separated by SDS/PAGE (12% acrylamide, 200:1 acrylamide/bisacrylamide), and proteins were detected by quantitative immunoblot (LI-COR Biotechnology) using a polyclonal antiserum raised against recombinant CA.

Fig. 1. Biochemical characterization of mutant virus-like particles used in this study. (A) Schematic illustration of the proteolytic cleavages involved in HIV-1 Gag maturation, ordered by relative rate as determined using purified PR in solution (23). Schematic representations of typical immature and mature viral morphologies are displayed to the left of the corresponding stages in the proteolytic cleavage cascade. (B) Schematic representation of Gag cleavage patterns in each of the cleavage mutants used. Mutated sites that cannot be cleaved are denoted with crossed out, dashed lines. Sites where cleavage occurs as in wild-type Gag are denoted by solid lines. Brackets under the schematics indicate the CA-containing products that remain unprocessed and their corresponding molecular mass. (C) SDS/PAGE analysis of virus preparations used for the structural analyses. Particles were purified from the supernatant of transfected HEK293T cells by ultracentrifugation through an iodixanol gradient. Samples were separated by SDS/PAGE (12.5% acrylamide, 30:1 acrylamide/bisacrylamide), and proteins were visualized by silver staining. Numbers to the left indicate the position of molecular mass standards (in kilodaltons). Purified recombinant Gag protein was used as a standard to estimate particle concentration. (D) Quantitative immunoblot analysis of MA-CA and MA-SP1 band intensities. Samples were separated by SDS/PAGE (12% acrylamide, 200:1 acrylamide/bisacrylamide), and proteins were detected by quantitative immunoblot (LI-COR Biotechnology) using a polyclonal antiserum raised against recombinant CA.

Despite the availability of detailed models for the immature and mature states, little is known about the intermediate states of HIV-1 maturation. The cleavages that release the domains of Gag proceed at very different rates, influenced by protein sequence and structure. In vitro, cleavage rates for different sites on the Gag polyprotein vary by a factor of ~400. Cleavage occurs fastest at the SP1-NC cleavage site, followed by SP2-SP1, MA-CA, NC-SP2, and, finally, CA-SP1 (23). Within the virus, computational models indicate that during the cleavage cascade, different sites are being cleaved in different Gag molecules at the same time (24). Interfering with cleavage at individual sites is disruptive to morphological maturation (22, 25–32).

We have previously investigated the structure of the CA layer in HIV-1 variants carrying combinations of cleavage site mutations within Gag to prevent cleavage at specific sites (30). These virus derivatives were named according to the CA-containing polypeptide present after proteolytic processing and included MA-SP1, MA-CA, CA-SP1, and CA-p6 (Fig. 1B). By determining low-resolution structures of the CA domain in each of these variants by cryo-ET and subtomogram averaging, we found that the immature lattice only disassembled in the case of CA-SP1, but remained intact when cleavage at either side of the CA-SP1 module was prevented. We concluded that combined cleavage both upstream of CA and downstream of the CA-SP1 module is required to destabilize the immature Gag lattice, and thus permit maturation (30). Once the immature lattice has been destabilized, the lower energy mature-like state is favored. When assembled in vitro, bypassing the immature state, CA, CA-SP1, and CA-NC constructs all preferentially assemble mature-like lattices (21, 33–35).

Despite this progress, many open questions remain about the mechanism of HIV-1 maturation. Does beta-hairpin formation or CA-SP1 helix disordering, or both, represent a structural switch that regulates maturation or a structural change that results from maturation? How do cleavages upstream and downstream of CA-SP1 together modulate the immature structure and lead to disassembly? What are the roles of individual cleavages? What do maturation intermediates look like?

Here, we have assessed the morphology of hundreds of virus particles, each from a panel of HIV-1 variants with cleavage site mutations, and have determined structures of their corresponding CA domain lattices at high resolution. These data allow minority phenotypes to be identified and local protein secondary structure to be resolved. Contrary to our previous results, this more detailed analysis revealed that cleavage on only one side of CA-SP1 can be sufficient to permit formation of the mature lattice and determined that CA-SP1 helix disordering, but not beta-hairpin formation, is a structural switch for HIV-1 maturation.

Results

Virus-like particles giving rise to the variants MA-SP1, MA-CA, CA-NC, and CA-SP1, respectively, were produced by transfection of subviral plasmids into HEK293T cells and purified as described previously (30). As described previously, all variants are noninfectious. Purity of particle preparations and Gag processing state were assessed by SDS/PAGE and quantitative immunoblot analysis of particle lysates using antibodies against CA (Fig. 1C and D). In all cases, the expected cleavage products were observed. As we had noted earlier (30), cleavage between CA and SP1 was incomplete in case of the MA-CA variant, occurring with roughly 50% efficiency in the preparation analyzed here (Fig. 1D). In the following, we first describe the overall morphology of the Gag lattice in the different variants; then, the high-resolution structures of the immature- and/or mature-like CA lattice observed in these particles; and, finally, the architecture of the capsid structures that were formed.

Morphology of the Gag Lattice in Cleavage Site Defective Particles.

Purified particles were vitrified by plunge-freezing, and their morphology was assessed by cryo-ET. Acquisition parameters for all variant particles are summarized in Table 1. At least 350 virus particles were imaged for all variants previously described as having immature-like morphology (MA-SP1, MA-CA, and CA-NC). For CA-SP1, 103 virus particles were imaged. Recent technical advances in cryo-ET (36) allowed us to generate higher quality tomograms, and to obtain approximately sevenfold larger datasets, than was possible in our earlier work (30), revealing the presence of minority phenotypes (Fig. 2B, i, ix and x, and C, xiv and xv).

As described previously (30), MA-SP1 particles displayed a striated layer beneath the viral membrane typical of the immature HIV-1 CA layer (Fig. 2A, i–v). Two percent (seven of 362) of virus particles displayed radially arranged densities corresponding to both CA and NC, indicating that they are fully immature and have not undergone proteolytic cleavage (Fig. 2A, i). Ninety-eight percent (355 of 362) of particles lacked the innermost NC ring (Fig. 2A, ii–v), consistent with proteolytic cleavage having occurred between CA and NC. Eighty-eight percent (310 of 355) of
the particles that had undergone cleavage contained a condensed luminal density characteristic of condensed RNP (Fig. 2A, ii–iv), as previously described for this variant (30).

In the MA-CA preparation, 3% (13 of 404) of particles had a fully immature phenotype (Fig. 2B, vi), while 84% (341 of 404) of particles displayed the morphology previously described for this variant: a striated immature-like CA layer similar to that commonly observed in MA-SP1 (Fig. 2B, vii and viii). The majority of these particles also contained a condensed RNP similar to MA-SP1 (discussed below). Eleven percent (45 of 341) of particles presented a CA layer that was thin, as is seen for the mature CA layer in wild-type HIV-1 virions (Fig. 2B, ix and x). The majority of these particles (34 of 45) contained a mixture of mature-like (thin) and immature-like (thick) CA layers (Fig. 2B, ix), while the remaining 11 particles appeared to contain only a mature-like thin CA lattice (Fig. 2B, x). In all cases, the mature-like CA layer had not formed a conical capsid but, instead, was close to the membrane (Fig. 2B, ix and x). In the 34 mixed-phenotype particles (Fig. 2B, ix), the condensed RNP complex was always adjacent to the mature-like part of the lattice. The presence of thin mature-like CA layers in MA-CA suggests that complete or partial structural maturation of the CA layer can occur in a stochastic manner without cleavage between MA and CA. In the majority of MA-CA and MA-SP1 particles, the immature CA layer (lacking the downstream RNP layer) had an architecture resembling a truncated sphere with a large gap. This architecture is typical of immature wild-type HIV-1 particles; the mature CA layer had not formed a conical capsid but, instead, was close to the membrane (Fig. 2B, ix and x). In the 34 mixed-phenotype particles (Fig. 2B, ix), the condensed RNP complex was always adjacent to the mature-like part of the lattice. The presence of thin mature-like CA layers in MA-CA suggests that complete or partial structural maturation of the CA layer can occur in a stochastic manner without cleavage between MA and CA.

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Structural Analysis of Immature-Like Gag Lattices. The morphologies of the HIV-1 variants described above suggest that partial structural maturation can take place despite incomplete proteolytic cleavage of Gag. To confirm this conclusion, and to obtain detailed structural information, we applied subtomogram averaging to determine higher resolution structures of the immature CA lattices identified in MA-SP1, MA-CA, and CA-NC particles (Fig. 3). The three datasets were processed independently as described in Materials and Methods to yield structures of the CA-SP1 layer with resolutions of 4.0 Å, 3.7 Å, and 4.5 Å, respectively (SI Appendix, Fig. S1A). We then fitted the Protein Data Bank (PDB) model previously generated based on the 3.9-Å structure of the immature Gag layer in wild-type immature HIV-1 particles (PDB ID code 5L93) (10) to the variant CA-SP1 structures. In all cases the PDB model could be fitted as a rigid body, with excellent correspondence between the model and density throughout the ordered region of the CA-SP1 layer (SI Appendix, Fig. S2).

Residues N-terminal to helix 1 of the CA-NTD, including residues 133–145 (CA residues 1–13, which form the N-terminal beta-hairpin in fully processed CA), were poorly resolved in all of the variant structures, as previously described for wild-type immature HIV-1 (10) (Fig. 3B). This observation suggests flexibility in this
Structural Analysis of Mature-Like Gag Lattices. We then applied subtomogram averaging to determine the structures of the mature-like CA lattices observed in MA-CA, CA-NC, and CA-SP1 particles at final resolutions of 8.3 Å, 9.7 Å, and 7.9 Å, respectively (Fig. 4 and SI Appendix, Fig. S1B). All three structures resembled that of the CA lattice determined in the mature wild-type HIV-1 capsid (11). They displayed the same arrangement of protein monomers within the lattice, suggesting that both intra- and interhexamer stabilizing interfaces are largely conserved in the variant particles (Fig. 4).

In the CA-NC structure, no density was seen for residues 354–378, corresponding to the helical region between the CA-CTD and NC (Fig. 4B). We conclude that the six-helix bundle observed in the immature lattice is absent in the mature CA-NC lattice, despite the abolition of cleavage between CA and SP1 and the presence of the SP1-NC regions downstream of CA.

The N-terminal beta-hairpin was clearly resolved in the CA-SP1 structure and appeared to adopt the closed conformation observed for the mature CA hexamer within wild-type capsids (11) (Fig. 4B). Density for the beta-hairpin was also visible for the CA-NC variant, but its conformation could not be reliably determined (Fig. 4B). In the CA-MA variant, formation of the beta-hairpin was prevented by covalent linkage of MA and CA. Consistently, we observed a much weaker density at the respective position compared with the other mature lattices (Fig. 4B); the observed density is likely to represent the initial residues of the linker connecting the CA-NTD to MA.

We next identified pentameric positions in the mature-like hexameric lattices of these variants, as we have previously described for wild-type mature HIV-1 capsids (11). For CA-SP1 particles, we

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**Fig. 2.** (A–D, i–xx) Representative viral morphologies for each of the cleavage mutants. Orthoslices through tomograms show representative examples of the viral morphologies observed in the different datasets. The frequency of each phenotype in the corresponding dataset is shown above each of the panels, as well as the percentage of the respective dataset that each absolute frequency represents. Color bars underneath each set of orthoslices represent the percentage of viral particles displaying immature (green), partially mature (yellow), mature (red), or undefined (black) morphology. Scale bars, 50 nm.

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region. We low-pass-filtered all structures to a resolution of 6 Å, allowing density N-terminal to helix 1 to be better resolved (Fig. 3C). In wild-type immature particles and in MA-SP1 and MA-CA variants, the resolved density corresponds approximately to Gag residues 143–149 (CA residues 11–17). In the CA-NC variant, this density was larger and wider than in the other cases. The size and shape of this density strongly suggest that the beta-hairpin has been formed within the immature Gag lattice in this case (Fig. 3C, Right), although the resolution achieved precludes a definitive statement.

The density resolved for the C-terminal CA-SP1 helix in wild-type immature HIV-1 extended to residue 371 of Gag (residue 8 of SP1) (10). This helix displayed the same length, with the same residues resolved, in the immature lattice of variants MA-SP1, MA-CA, and CA-NC (Fig. 3D), indicating that cleavage between SP1 and NC does not result in structural change in the CA-SP1 helical bundle when the CA-MA site remains uncleaved. MA-CA particle preparations contained a mixed population of 20A and MA-MA particles, as shown by immunoblot, with ∼50% of molecules uncleaved at the CA-SP1 processing site (Fig. 1D). The distribution of cleaved and uncleaved molecules between individual particles or between hexamers within one particle cannot be derived from this bulk analysis, however; we observed no structural change in the helix for the CA-MA variant, despite partial removal of SP1.

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**Fig. 3.** (A) Density maps of the N-terminus of MA-CA (MA residues 1–10), CA-NC (CA residues 11–17), and CA-SP1 (SP1 residues 1–8) hexamers in the immature lattice of wild-type HIV capsids. The densities range from the N-terminus (green) to the C-terminus (red). Filled and open circles represent mono- and multimers, respectively. Scale bars, 50 nm. For the CA-SP1 density, the C-terminus is ambiguous (black). (B) The equivalent density maps are shown for mature MA-CA (MA residues 1–10), CA-NC (CA residues 11–17), and CA-SP1 (SP1 residues 1–8) hexamers. Scale bars, 50 nm. (C) The density maps of the N-terminus of CA-MA (MA residues 1–10), CA-NC (CA residues 11–17), and CA-SP1 (SP1 residues 1–8) hexamers in the immature lattice of wild-type HIV capsids. The densities range from the N-terminus (green) to the C-terminus (red). Filled and open circles represent mono- and multimers, respectively. Scale bars, 50 nm. For the CA-SP1 density, the C-terminus is ambiguous (black). (D) The equivalent density maps are shown for mature CA-MA (MA residues 1–10), CA-NC (CA residues 11–17), and CA-SP1 (SP1 residues 1–8) hexamers. Scale bars, 50 nm.
determined 11 pentameric positions. All of these pentamers were detected in regions with high lattice curvature. The low number of pentameric positions compared with wild-type capsids reflects the fact that only incomplete polyhedral structures are seen for this variant, and that they have generally low overall curvature. For CA-NC, we identified 14 pentamers, again at positions of high curvature. For MA-CA particles, we identified only two pentametrically coordinated positions, which was insufficient for structure determination. For both CA-SP1 and CA-NC, we extracted subtomograms from pentametrically coordinated positions and iteratively averaged and aligned them as previously described (11) to generate low-resolution reconstructions of the CA pentamers (Fig. 5). An estimate of the resolution achieved was obtained by calculating the Fourier shell correlation (FSC) between the averages and the structure previously obtained for the HIV-1 wild-type pentamer (Electron Microscopy Data Bank accession code EMD-3466) (SI Appendix, Fig. S1C), resulting in 24 Å for CA-SP1 and 22 Å for CA-NC according to the 0.5 FSC criterion. Up to the determined resolutions, the structures of the pentamers from mutant particles were consistent with the structure of the wild-type pentamer.

Architecture of Immature and Mature CA Lattices. We next analyzed the architecture of the CA lattices within the variant particles in comparison to wild-type immature and mature particles. To do this, we visualized the global arrangement of the CA lattice using previously described “lattice maps” (9) (Fig. 6). For all variants, the arrangement of immature-like Gag lattices was generally consistent with what had been observed for wild-type particles: The lattices were hexameric and contained small gaps and imperfections as well as one larger gap in the lattice that likely corresponds to the site where budding occurred. The lattice maps also confirmed the observation made above based on visual analysis of the tomograms that for a small subset of MA-CA and MA-SP1 particles, there was no large gap and often no RNP (Fig. 2A, v and B, viii).

Lattice maps derived from mature assemblies found in MA-CA, CA-NC, and CA-SP1 particles confirmed the morphological analyses described in the previous section. Of the 78 CA-SP1 particles containing mature lattices, three appeared to be almost closed structures with irregular morphology. The remaining 75 represented open, incomplete polyhedra, containing cracks and gaps as well as pentametrically coordinated positions (Fig. 6A, xiv; B, xxv; and C, xxvi).

In the case of the MA-CA variant, the extent of the lattice with mature morphology varied between particles. Consistent with CA being connected to the MA/membrane layer, the hexameric lattice displayed a low curvature following the viral envelope (Fig. 6A, xiii; B, xiv; and C, xv). Cracks and gaps were detected in this lattice, similar to those observed in immature Gag lattices; these imperfections can relieve structural stress that would otherwise build up in a curved hexameric lattice. In contrast, pentamers were almost completely absent in these particles. Although we cannot exclude the possibility that the absence of the CA beta-hairpin inhibits pentamer formation in the MA-CA variant, we suggest that the absence of pentamers results from the lack of regions of high curvature in this case.

In CA-NC particles, mature lattices often formed irregular polyhedral cores more variable in morphology than those seen in wild-type mature viruses. From the 35 CA-NC virions displaying mature lattices, four of the virions contained two cores. Twenty-four of the 39 observed mature lattices formed irregular polyhedra, two appeared conical, and two appeared triangular. All of these phenotypes have previously been reported for wild-type mature cores (6, 7). The remaining mature lattices (11 of 39) formed open, curved sheets that were smaller in size than the more complete polyhedral cores.

Where immature and mature lattices were found in the same particle, they abutted each other (Fig. 6A, x and xiii; B, xi and xx; and C, xii and xvi). They were connected by a region of apparent local disorder, as suggested by lack of alignment/correspondence to either mature or immature CA references. Immature and mature
lattices have also been found within the same particles after treatment with the maturation inhibitor PF-46396 (40), but these lattices do not abut each other. The close spatial proximity of the two lattice types in the cleavage mutants suggests that they are linked, either through interactions with the membrane via MA (for MA-CA) or through interactions with the RNP (for CA-NC).

**Discussion**

Formation of the wild-type, mature HIV-1 core requires maturation at three levels: (i) proteolytic maturation—correct processing by PR at all five sites in Gag, (ii) structural maturation—transition of the CA packing arrangement from an immature to mature CA lattice, and (iii) architectural maturation—transition from a truncated spherical immature Gag layer with irregular defects to a closed conical mature core with pentamers at the narrow and wide ends. Previous studies on viral cleavage site mutants (22, 25–32) have shown that interfering with the degree or rate of proteolytic cleavage at individual sites in Gag almost invariably leads to defects in the architectural transition manifested in irregular core shapes, incomplete cores, or empty cores that exclude the viral genome. Our observations here support and refine these results: We found that all variants with cleavage site mutations, and therefore defective in proteolytic maturation, were defective in architectural maturation. On the other hand, some variants with cleavage site mutations do support (partial) structural maturation of the CA lattice, thus shedding light on the regulation of this process.

Consistent with previous results (30), we observed that cleavage both upstream and downstream of the CA-SP1 module is sufficient for structural maturation of the CA lattice. Based on lower resolution structures from smaller datasets (30), we had previously concluded that cleavage both upstream and downstream of the CA or CA-SP1 lattice is necessary for structural maturation, but this interpretation needs to be revised based on the results presented here. Although predominantly immature, both MA-CA and CA-NC particles did contain regions of mature CA lattice, indicating that cleavage at both termini of CA is not essential for CA structural maturation. MA-SP1 particles, however, appeared to be entirely immature, suggesting that cleavage exclusively between CA and NC is only sufficient for structural maturation when it occurs between CA and SP1. These observations indicate that the relationship between proteolytic maturation and structural maturation is complex: There is no single cleavage site at which successful proteolytic cleavage is required for structural maturation; when any cleavage site between MA and NC is blocked by mutation, a combination of other cleavages can permit structural maturation, albeit at low efficiency.

There has been a debate in the field as to whether HIV-1 maturation proceeds by disassembly/reassembly or via a displacive transition. A fully displacive transition (i.e., direct transformation of the immature lattice into the mature lattice) (41) is inconsistent with the differences in the relative relationships and numbers of CA in the immature and mature lattices. We and others therefore prefer a model in which the lattice disassembles into smaller oligomers or patches, which undergo structural maturation, followed by assembly of the mature capsid architecture (20, 42). In the context of this model, the observed defects in architectural maturation in the MA-CA and CA-NC mutants can simply be caused by CA remaining tethered to MA or NC during structural maturation of the CA lattice. The architectural maturation defect observed for CA-SP1 suggests that dysregulation of disassembly kinetics can also lead to defects in mature lattice architecture. The observation of distinct and segregated patches of mature and immature lattices in both MA-CA and CA-NC mutants, as well as in particles treated with the maturation inhibitor PF-46396 (40), suggests that structural maturation occurs in a processive manner where the likelihood of maturation is increased by maturation of a neighboring hexamer. This could simply result from local destabilization of the structure by cleavage of neighboring molecules. We note that whether maturation is processive or stochastic has no bearing on whether it occurs via disassembly/reassembly or via a displacive transition.

We observed that the immature CA-NC lattice can accommodate the N-terminal beta-hairpin of CA. Conversely, MA-CA can form a mature CA lattice lacking the beta-hairpin, consistent with the ability of a construct lacking the N-terminal 12 residues of CA to assemble a mature CA lattice in vitro (43). These findings indicate that beta-hairpin formation is neither sufficient to destabilize the immature CA lattice nor required to assemble the mature lattice. This makes it unlikely that formation of the beta-hairpin is the switch controlling structural maturation of CA. Instead, it suggests that the hairpin may be functionally relevant after maturation rather than influencing the process of maturation itself. Conceivably, beta-hairpin formation may influence the properties of the adjacent central pore in the CA hexamer that appears to be important in early replication stages (44).
In all immature lattice structures observed, the CA-SP1 bundle was intact and had the same length. Conversely, no helical density was visible downstream of the CA-CTD in any of the mature lattice structures, including that of CA-NC, indicating that the CA-SP1 region is disordered in all these cases. Consistent with these observations, in mature-like CA-SP1 tubes assembled in vitro, the predominant conformation is nonhelical and no six-helix bundle is present (45, 46). Destabilization of the CA-SP1 six-helix bundle therefore correlates with structural maturation of the CA domains, even in the absence of proteolytic cleavage at the CA-SP1 site.

In the MA-CA variant, partial cleavage takes place between CA and SP1. Since helix destabilization is required to allow PR to access the cleavage site, this implies that the CA-SP1 helix can undergo transient destabilization when CA remains tethered to MA, even though the six-helix bundle is predominantly present. In the MA-CA variant, PR cleavage makes the transient destabilization permanent, and some regions of mature CA lattice are therefore formed. In the MA-CA variant, cleavage between CA and SP1 cannot take place, and no regions of mature lattice are observed. Despite cleavage of a subset of CA-SP1 sites in the MA-CA variant, we do not see a structural change in the six-helix bundle in the immature lattice. We see three possible causes for this: (i) molecules that have been cleaved between CA and SP1 have undergone structural maturation and form part of the mature-like lattice in the mixed-phenotype particles, (ii) partially cleaved six-helix bundles largely retain their structural integrity, or (iii) complete cleavage of a subset of six-helix bundles does not destabilize the immature lattice. It is likely that a combination of these causes explains our observations.

Structural maturation involves resolution of interactions that stabilize the immature Gag lattice and formation of a stable, mature CA lattice. Destabilization of the immature lattice is a prerequisite for structural maturation. Several structural features contribute to stabilization of the immature lattice: (i) CA–CA interactions, (ii) interactions within the helical bundle formed at the CA-SP1 boundary, (iii) tethering of the CA lattice via C-terminal NC–RNA interactions, and (iv) tethering of the CA lattice by N-terminal MA–membrane interactions. Based on our results, we suggest that resolution of the CA-SP1 six-helix bundle represents the switch for structural maturation. This resolution could be achieved by removal of the tethering interactions to MA and/or NC, or by the transient formation of destabilizing interactions, for example, by formation of the beta-hairpin within the immature lattice. The observations that (i) no individual cleavage site is necessary for CA-SP1 maturation and (ii) beta-hairpin formation does not correlate with structural maturation strongly argue against a role of transient destabilizing interactions. Instead, the immature lattice must be resolved by loss of stabilizing interactions, such as tethering via MA to the membrane and/or through NC to RNA. Loss of both of these tethers, as in the CA-SP1 variant, leads to destabilization of the six-helix bundle and structural maturation. Loss of the tether to MA leads to partial structural maturation, as does loss of the tether to NC in the MA-CA variant. Both of these tethers therefore contribute to stability of the immature lattice.

In summary, the data described here are consistent with a model in which integrity of the six-helix bundle within the immature CA lattice is maintained by the cumulative effects of multiple stabilizing interactions. Structural maturation is promoted by removing various subsets of these interactions through proteolytic cleavage, thereby destabilizing the bundle. In keeping with this model, maturation can be inhibited by stabilizing the helical form of SP1. This can be achieved by specific point mutations, such as threonine to isoleucine at position 8 in SP1 (47). Alternatively, treatment with maturation inhibitors, such as bevirimat, stabilizes the CA-SP1 bundle and also arrests the virus in an immature-like state (39). Accordingly, resistance against maturation inhibitors develops through mutations that destabilize the six-helix bundle and thereby counteract the stabilizing effect of the inhibitor, thus restoring the maturation switch (10, 17, 48, 49).

Materials and Methods

Particle Production and Purification. Virus-like particles were produced in HEK293T cells transfected with plasmids derived from pCHIV (50), which carried the indicated PR cleavage site mutations (29, 51). For this, cells seeded in 175-cm² flasks were transfected with 70 μg of plasmid per flask using a standard CaPO₄ transfection procedure. At 36 h posttransfection, tissue culture supernatants were harvested and filtered through 0.45-μm nitrocellulose, and particles were concentrated by ultracentrifugation through an iodixanol gradient as described previously (52). Particle-containing fractions were diluted with PBS (1:8) and again concentrated by ultracentrifugation (30 min, 44,000 rpm in a Beckman SW60 rotor; Beckman Coulter Life Sciences). Particle pellets were gently resuspended in PBS, fixed with 1% paraformaldehyde for 1 h at 0 °C, and stored in aliquots at −80 °C.

Immunoblot Analysis. Particle lysates were separated by SDS/PAGE (200:1 acrylamide:bisacrylamide), and proteins were transferred to a nitrocellulose membrane by semidy blotting. Gag-derived proteins were detected by quantitative immunoblot analysis on a LI-COR Odyssey CLx infrared scanner (LI-COR Biotechnology) following the manufacturer’s instructions, using rabbit polyclonal antiserum raised against recombinant HIV-1 CA and IRDye 800CW donkey anti-rabbit IgG secondary antibody (LI-COR Biotechnology). ImageStudio software (version 5.2; LI-COR Biotechnology) was used for generation of images and quantitation of band intensities.

Cryo-EM Sample Preparation. A solution of PBS containing 10-nm-diameter colloidal gold beads was added to a suspension of purified HIV-1 mutant particles with a final ratio, by volume, of 1:1 between the gold suspension...
and viral suspension. A portion (2.5 µL) of the sample was applied to a C-flat 2/2 3C grid previously glow-discharged at 20 mA for 30 s. Samples were blotted and plunge-frozen in liquid ethane using an FEI Vitrobot Mark II operated at 15 °C and 100% humidity. The grids were stored in liquid nitrogen until image acquisition.

Cryo-ET Tomography. Tomographic tilt series between −60° and +60° were acquired using a dose-symmetric tilt scheme (53), with a tilt increment of 3°, on an FEI Titan Krios transmission electron microscope at 300 kV in super-resolution mode, equipped with a Gatan Quantum 967 LS energy filter with a slit width of 20 eV. Tilt series images were acquired on a Gatan K2xep detector with 10 frames per tilt and a total dose of −150 eV/Å² across all of the tilts. Superresolution frames were aligned on-the-fly in SerialEM (54) and Fourier-cropped to 3,708 × 3,708 pixels, giving a final pixel size of 1.35 Å per pixel in the unbinned image stacks.

The tilt image stacks were sorted by tilt angle using IMOD (55), and exposure filtering was performed in MATLAB (MathWorks) using the formula described by Grant and Grigorieff (56). Contrast transfer function (CTF) determination was performed on the non-exposure-filtered stacks using CTFFIND4 (57). All tomograms were then reconstructed using IMOD with 5° in-plane search, sixfold symmetry, and 29.9-Å low-pass filter. For MA-SP1, five iterations were performed while applying a 29.9-Å low-pass filter. For MA-CA and CA-NC, seven iterations were performed with a 32.4-Å low-pass filter. The average was shifted to center the sixfold symmetry axis in the box, and two further iterations of alignment were performed with the same parameters, but with a sixfold symmetrized reference. Subvolumes closer than 32.4 Å (four pixels) apart were removed from the dataset, along with those with a low cross-correlation coefficient (CCC), which did not contain Gag. For MA-SP1, one final iteration of alignment was then performed on the remaining subvolumes in the dataset, with a 4 × 1° cone search, 5 × 1° in-plane search, sixfold symmetry, and 29.9-Å low-pass filter. For MA-CA and CA-NC, three further iterations of angular search were performed with the same parameters as before and with sixfold symmetry. The resulting averages here were used as the initial references for each dataset.

For alignment of each full dataset against the respective reference, 2D CTF-corrected tomograms were used unless otherwise stated. Subtomograms were extracted from the full set of 8x binned tomograms as before. The respective reference was used for two iterations of alignment with a 7 × 6° angular search range for each Euler angle and a 32.4-Å low-pass filter. As before, points with a low CCC and those closer together than half the lattice spacing of 8 nm were then removed from the dataset. The positions and orientations of the points obtained from alignment of 8x binned subtomograms were then used for successive alignment using 4x binned, 2x binned, and then unbinned subtomograms using the same steps and parameters as previously described (10), with the data split into independent odd and even half-datasets after alignment of the 4x binned subtomograms.

For 3D CTF estimation and correction, after two iterations of alignment of unbinned subtomograms from 2D CTF-corrected tomograms, the positions of all points were used to calculate the center-of-mass for defocus estimation of each dataset. These values were used for reconstruction of the unbinned tomograms with 3D CTF correction as described above. Subtomograms with a box size of (259 Å)³ were then reconstructed from the 3D CTF-corrected tomograms and averaged to produce new odd/even references for each half-datasets. A further iteration of alignment was run against these references with a 2 × 1° angular search for all Euler angles and a band-pass filter of 8.1–25.9 Å applied to the references during alignment. Image processing statistics are summarized in Table 2.

Image Processing: Immature Viruses. For the MA-SP1, MA-CA, and CA-NC datasets, the centers of immature viruses in 8x binned tomograms were marked manually using a custom plug-in for UCSF Chimera (60). An oversampled grid of initial extraction points was defined using MATLAB on a spherical surface centered on these positions, with a radius set appropriately for each virus. Subtomogram averaging and alignment were performed independently for each dataset using MATLAB scripts based on TOM (61), AV3 (62), and Dynamo (63) as previously described (10). For each dataset, viruses from one tomogram with high defocus (>4.0 µm) were chosen for construction of an initial reference.

For construction of initial references, subtomograms were extracted from 8x binned tomograms with a box size of (389 Å)³ and averaged. Iterative reference-free alignment was performed with a 6 × 4° conical search and 8 × 5° in-plane search. For MA-SP1, five iterations were performed while applying
Table 2. Image processing statistics for all cleavage mutants

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circular Gaussian kernel of 20 pixels in MATLAB using the “tom_filter” function of the TOM package and utilized to generate isosurfaces. Points with a spacing of two pixels were sampled along the isosurfaces at an intensity threshold of 0.3 and were assigned Euler angles based on their orientation relative to the normal of the surface at each point. The in-plane angle of each point was randomized between 0° and 360°.

For the CA-SP1 and MA-CA viruses containing mature lattices, initial extraction points along the mature CA lattice were defined in Amira (version 4.1.2), by marking the center and measuring the radius of a sphere matching the lattice curvature. Extraction points were generated in MATLAB as for the immature lattice, with a spacing of 2.16 nm (two pixels). Initial CA-SP1 and MA-CA references were constructed by extracting subtomograms from a single 8x binned tomogram from each dataset with a box size of (691 Å)³. For CA-NC, only a single viral core was used to generate the reference. Subtomograms from each respective dataset were averaged, and reference-free alignment was run independently with a 2 × 10° cone search, 18 × 10° in-plane search, 49.4-Å low-pass filter, and no symmetry applied until each reference converged on a hexagonal lattice. Two further iterations of alignment with the same parameters were run, but with a manually determined threshold for exclusion of points with low CCC from the average. The references were shifted to center them on the sixfold symmetry axis of a CA hexamer, and each alignment reference was generated by running two iterations of alignment with a 5 × 4° cone search, 5 × 6° in-plane search, 49.4-Å low-pass filter, and sixfold symmetry.

The full CA-SP1 and MA-CA mature hexamer datasets were processed as follows. Initially sampled points, as described above, were used for extraction of subtomograms from 8x binned tomograms of each respective dataset. Subtomograms from each dataset were subjected to two initial iterations of alignment against the respective reference generated as above, with 10 × 4° cone search, 10 × 3° in-plane search, 49.4-Å low-pass filter, and sifx symmetry applied to the reference. Misaligned and duplicate points from oversampling were then removed from the dataset based on CCC and with a minimum pairwise distance threshold of 5.4 nm. For the CA-SP1 dataset, two more iterations of alignment were run after this cleaning step.

The full CA-NC dataset was processed differently due to the irregular surface shapes of its variable mature cores. All CA-NC alignments were run with sixfold symmetry applied to the reference. To correct for inaccuracies in the initial tracing of the CA-NC cores, subtomograms were extracted from 8x binned tomograms as for MA-CA and CA-SP1 and aligned along the z axis only against the reference for two iterations, with a 4 × 10° cone search, 3 × 10° in-plane search, and 49.4-Å low-pass filter. Subtomograms with low CCC were removed, and remaining points were converted into a UCSF Chimera marker set. Each marker set was overlaid on the corresponding tomogram in UCSF Chimera, and gaps in the lattice of points were filled using the Volume Tracer tool if necessary. These marker sets were read into MATLAB and used to generate an isosurface for resampling as before. Using the new surfaces, subtomograms were extracted from 8x binned tomograms and aligned as before. After cleaning based on distance and CCC as before, the subtomograms were subjected to two further iterations of alignment with a 4 × 5° cone search and 5 × 6° in-plane search.

For all mature datasets, after alignment from 8x binned data, subtomograms were extracted with a box size of (346 Å)³ from 4x binned tomograms at the aligned positions and averaged to produce a reference, against which the subtomograms were aligned for three iterations with a 4 x 6° search for each Euler angle and a low-pass filter of 34.6 Å. Subtomograms were then extracted from 2x binned tomograms with a box size of (346 Å)³, and those with gray values more than ±1 SD from the mean (which may contain artifacts from gold bead) were removed. The remaining subtomograms were split into separate odd/even half-datasets and averaged to produce odd/even alignment references. Subtomograms in each half-dataset were aligned against the respective reference over two iterations with a 3 × 2° search for all Euler angles and a low-pass filter of 24.7 Å. The positions from these alignments were used to extract subtomograms of (346 Å)³ from the unbinned tomograms, which were averaged to produce new odd/even references. One iteration of alignment with a 3 × 1° angular search for all Euler angles and 15.3-Å low-pass filter was run, followed by one iteration of alignment with a 2 × 1° angular search for all Euler angles and bandpass filter of 13–20 Å. Aligned subtomograms were then extracted from the unbinned 3D CTF-corrected tomograms and averaged to produce new references, which were used to repeat the last alignment iteration.

Visualization of Structures and Maps. Immature CA lattices were analyzed by fitting the central monomer from PDB ID code 5L93 as a rigid body using UCSF Chimera. Mature CA lattices were analyzed equivalently, fitting a monomer from PDB ID code 4XF1. To compare the CA-NTD density with structures with and without a beta-hairpin, we fit PDB ID code 5HKG and PDB ID code 1Len (model 4) as rigid bodies.

Isosurface renderings of each final electron density map were produced using UCSF Chimera, as were orthoslics of the final pentamer structures obtained from 4x binned data. The fit of the immature Gag atomic model (PDB ID code 6L93) into each of the immature cleavage mutant hexamer maps was visualized using PyMOL (version 1.8.6.0; Schrödinger, LLC). The wild-type pentamer is visualized using a structure obtained from 4x binned data to facilitate a direct comparison. Orthoslics of viruses, as shown in the morphology figures, were generated by applying a 3D Gaussian filter with a five-pixel kernel to 4x binned tomograms in MOLM and were rendered at a thickness of 5.4 Å in UCSF Chimera.

The positions and orientations of aligned subtomograms were visualized using lattice maps, which consist of an appropriate geometric object placed at the coordinates of each subtomogram box center, and rotated by the Euler angles found by subtomogram alignment. To visualize lattice morphology (Fig. 6 and C), we used the coordinates from the 8x binned alignments after cleaning based on distance and CCC. The CCC threshold for visualization was set per virus such that only the subset of clearly misaligned subtomograms (those with translations or rotations that shifted/rotated them away from any underlying CA protein layer) was excluded. This ensured that the lattice maps were as complete as possible. We then manually excluded a small number of subtomograms that were retained above the CCC threshold but that, by visual inspection, were clearly misaligned or were not continuous with any region of hexameric lattice. For particles containing both immature and mature lattices, alignments with mature and immature references were considered independently. The references are sufficiently distinct that we did not identify positions at which subtomograms aligned to both references. All lattice map analysis was performed using a custom plug-in for UCSF Chimera.

Data Availability. The structures described in this paper have been deposited in the Electron Microscopy Data Bank (www.ebi.ac.uk/pdbe/emdb) under accession codes EMD-0164-EMD-0171 (Table 2).
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