The Structural Biology of HIV Assembly

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Abstract

HIV assembly and replication proceed through formation of morphologically distinct immature and mature viral capsids that are organized by the Gag polyprotein (immature) and by the fully processed CA protein (mature). The Gag polyprotein is composed of three folded polypeptides (MA, CA, and NC) and three smaller peptides (SP1, SP2, and p6) that function together to coordinate membrane binding and Gag-Gag lattice interactions in immature virions. Following budding, HIV maturation is initiated by proteolytic processing of Gag, which induces conformational changes in the CA domain and results in assembly of the distinctive conical capsid. Retroviral capsids are organized following the principles of fullerene cones, and the hexagonal CA lattice is stabilized by three distinct interfaces. Recently identified inhibitors of viral maturation act by disrupting the final stage of Gag processing, or by inhibiting formation of a critical intermolecular CA-CA interface in the mature capsid. Following release into a new host cell, the capsid disassembles and host cell factors can potently restrict this stage of retroviral replication. Here, we review the structures of immature and mature HIV virions, focusing on recent studies that have defined the global organization of the immature Gag lattice, identified sites likely to undergo conformational changes during maturation, revealed the molecular structure of the mature capsid lattice, demonstrated that capsid architectures are conserved, identified the first capsid assembly inhibitors, and begun to uncover the remarkable biology of the mature capsid.

Keywords

HIV; Gag; CA

Introduction

HIV-1 assembles into morphologically distinct immature and mature virions. The virus initially assembles and buds from cells as a noninfectious, immature spherical particle that is organized

by a layer of Gag proteins that are associated with the inner viral membrane (Fig. 1). Upon budding, immature particles dramatically rearrange to form mature, infectious virions. The mature capsids are mostly conical, though a small percentage of cylindrical capsids are also observed. The remarkably distinct morphologies of the immature Gag lattice and the capsid lattice presumably reflect specific requirements for organizing viral assembly in the producer cell and coordinating viral replication in the new host cell (Fig. 1a) (reviewed in [1-4]). The capsid and its contents are called the "core," and this is the object that is released into the cytoplasm of a newly infected cell to initiate a new cycle of viral replication [3-8].

The structural proteins of HIV-1 are all derived from the Gag polyprotein (Fig. 1b), which is myristoylated co-translationally. Gag molecules assemble at the plasma membrane, and immature virions acquire a lipid envelope as they bud (Fig. 1a, d, and f). The viral protease is activated during assembly, and it cleaves Gag to generate a set of new proteins and spacer peptides (SP), termed MA, CA, SP1, NC, SP2, and p6. These newly processed proteins then reassemble to form the distinct layers of the mature virion: MA remains associated with the inner viral membrane (the "matrix" layer), NC coats the viral RNA genome (the "nucleocapsid" layer), and CA assembles into the conical capsid that surrounds the nucleocapsid and its associated enzymes, reverse transcriptase (RT) and integrase (IN) (Fig. 1e and g). High-resolution tertiary structures of the MA, CA, and NC proteins from a number of different orthoretroviruses show that the folds of the individual Gag domains are highly conserved, despite limited primary sequence conservation (Fig. 1c and reviewed in [9,10]). It can therefore be inferred that they share the same basic architecture, despite significant variations in capsid shape that were historically used to define different retroviral genera.

Gag as an assembly machine

The immature virion is a roughly spherical shell of radially extended Gag molecules. The N-terminal Gag MA domains are bound to the inner viral membrane, and the C-termini of the Gag molecules project into the center of the virus (Fig. 1 and 2). To a first approximation, all of the information necessary for retroviral particle assembly resides in the Gag polypeptide. For example, Gag alone can form extracellular virus-like particles in the absence of other viral proteins [11] and Gag molecules can spontaneously assemble into spherical, immature virus-like particles *in vitro* [12-14]. Nevertheless, although Gag itself encodes the necessary tertiary and quaternary interactions, it must be emphasized that assembly requires nonspecific RNA interactions both *in vivo* and *in vitro*, and is assisted by host factors *in vivo*, including trafficking factors, assembly chaperones, and the ESCRT budding pathway, as reviewed elsewhere [15-18].

Although it was appealing to imagine that the Gag molecules were organized with icosahedral symmetry, analyses of immature virions of HIV-1 [19-21], Rous sarcoma virus (RSV) [22] and murine leukemia virus (MLV) [23] revealed non-icosahedral paracrystalline lattices composed of hexameric Gag rings. The rings are close-packed, with characteristic inter-ring spacings of ~80 Å. The immature virions of HIV-1 particles are often discontinuous, implying that the Gag lattice need not close fully during virus budding and that the membrane also plays an important role in stabilizing immature particles ([24], H.-G. Kräusslich, pers. comm.). The different regions of Gag must, of course, work together in concert to drive particle assembly, and the emerging picture is that many different interactions collaborate to create viral particles. Indeed, the requirement for multiple weak, but highly cooperative interactions probably provides a mechanism for ensuring that particle formation only occurs when all essential aspects of assembly such as membrane binding and RNA packaging have been met. Thus, while different regions of Gag contribute to membrane binding, Gag-Gag interactions and cofactor recruitment, we wish to emphasize that the Gag polypeptide is an assembly machine that simultaneously performs *and integrates* all of these different activities.

Membrane binding is mediated primarily by the MA domain

MA binds directly to the inner leaflet of the plasma membrane, and recent studies have helped elucidate how this domain targets Gag to the plasma membrane and triggers particle assembly at the correct time and place (Fig. 2a and b) [16]. HIV-1 MA (colored red in Fig. 1 and 2) is a helical domain that displays a conserved patch of basic residues on the same face as the N-terminal myristoyl modification. C-terminal MA residues form an extended α-helix that projects away from the globular domain, on the side opposite the membrane binding face [25,26]. Membrane binding is mediated by insertion of the myristoyl group into the lipid bilayer and by the basic patch, which binds acidic phospholipids, particularly $PI(4,5)P_2$, a phosphoinosotide that is concentrated in the plasma membrane (Fig. 2a and b) [27-31]. Importantly, HIV-1 Gag is incorrectly directed to internal membranes upon depletion of cellular $PI(4,5)P_2$, indicating that this phospholipid is required for proper targeting *in vivo* [28]. Binding studies indicate that ionic interactions dominate the energetics of HIV-1 MA membrane binding *in vitro* and that the myristoyl group makes only modest contributions to the binding interaction [32]. Indeed, some retroviral MA proteins lack myristoyl (or similar) modifications altogether, and in those cases, membrane binding is mediated exclusively by ionic interactions [33,34]. Cholesterol also plays an important role in Gag targeting and particle assembly [35], but the molecular mechanism by which Gag senses cholesterol is not yet known.

MA membrane binding appears to be positively coupled to Gag oligomerization and assembly in several different ways. Locally, the MA domain exhibits a two-state, "myristoyl switching" mechanism in which the myristoyl group is sequestered in a groove along the body of the globular domain in the soluble form of the protein and is then exposed for insertion into the bilayer [36-39]. Myristoyl exposure is favored by $PI(4,5)P_2$ binding, providing a mechanism for coupling plasma membrane recognition and membrane binding [40]. Stable membrane binding and myristoyl exposure are also favored by Gag oligomerization, [32,37,41,42], and inositol phosphate binding promotes trimerization of Gag fragments even in the absence of the myristoyl moiety [43]. Thus, downstream Gag-Gag interactions contribute to the affinity of MA-membrane interactions (and vice versa). The MA domain may also negatively regulate Gag assembly, probably by "folding back" onto the NC region, providing yet another potential mechanism for coupling membrane binding with the formation of the lateral Gag-Gag interactions essential for immature virion assembly [43,44].

Gag-Gag lattice interactions in the immature virion are mediated primarily by CA and SP1

The immature virion is stabilized by lateral interactions distributed throughout the Gag polypeptide, and the historical view of a discrete interaction site (or "I" domain) is therefore an oversimplification. Nevertheless, specific Gag regions are particularly important for immature particle formation, whereas other regions, such as MA and p6, are largely dispensable. Although MA couples membrane binding and assembly, it does not form a continuous regular lattice in the immature virion [22] and is dispensable for particle formation [45]. Similarly, p6 recruits the cellular machinery required for virus budding [46], but does not make important Gag-Gag contacts. In contrast, critical contacts are made by the C-terminal domain of CA (CA_{CTD}), the adjacent SP1 spacer, the NC region, and, to a lesser extent, the N-terminal domain of CA (CA_{NTD}) [42,47-52].

A recent electron cryotomographic analysis of immature HIV-1 virions confirmed the radial "beads on a string" arrangement of the different Gag domains and showed that the CA and SP1 regions formed a close-packed lattice of cup-shaped hexamers, in which the walls and bottom of the cups appeared to correspond to the CA and SP1 layers, respectively (Fig. 2c and d) [24]. In contrast, the MA and NC layers lacked hexagonal order. Although the resolution was insufficient to position domains unambiguously, this reconstruction can now be used to guide

possible models for Gag-Gag interactions, as constrained by relevant biochemical and high resolution structural data.

High resolution structures show that the free MA and CA_{NTD} domains are connected by a flexible linker that could easily span the distance between the MA and CA_{NTD} layers of the immature virion [53,54]. The MA domain does not change structure when tethered to CA_{NTD}, consistent with the idea that the membrane-binding "heads" of MA are connected via flexible linkers to the CA_{NTD} hexamers below. The oligomeric state of these membrane-bound MA proteins has not yet been established, however, since the matrix layer does not form a continuous lattice [22], and MA proteins can form both trimers [25,40,43,55] and hexamers [56] *in vitro*. Interestingly, the MA and CA domains of RSV Gag are separated by an extended linker, called p10, which contributes to Gag assembly by forming an α-helical bundle with the neighboring subunit in the Gag hexamer ([57], V. Vogt, pers. comm.).

The CA_{NTD} domain (colored dark green in Figs. 1-3) is composed of seven α-helices packed in the shape of an arrowhead (CA helices 1-7), with an extended loop connecting helices 4 and 5 that binds the prolyl isomerase, cyclophilin A. CA_{NTD} interfaces within the immature lattice are still a matter of speculation, but biochemical studies have identified two regions that are important for immature virion formation: one encompassing helices 1 and 2, and a second surrounding helices 4 and 7 [42,47,58,59]. These observations are consistent with models in which the hexameric rings of CA_{NTD} in the immature lattice are stabilized by six-fold symmetric interactions involving helices 1 and 2, and contact(s) across the local two- or three-fold axes between neighboring hexamers involving helices 4 and 7 [24]. Indeed, it was recently proposed that similar interhexamer contacts seen in two different MLV CA_{NTD} crystal forms might mimic the three-fold symmetric contacts between Gag hexamers in the immature lattice, and this proposal was supported by mutational analyses [60,61]. Nevertheless, further studies will be required to confirm these models and provide molecular details of the intra- and interhexamer contacts in the immature HIV-1 Gag lattice. In this regard, considerable insight will likely be gleaned from higher resolution structural studies of immature HIV-1, MLV, and MPMV (Mazon-Pfizer monkey virus) Gag assemblies formed *in vitro* [62-65], and from deuterium-exchange protection experiments that map protein-protein interfaces in the immature lattice (P. Prevelige, pers. comm.).

The CA_{CTD} is also critical for immature particle assembly, and it appears to make both inter- and intrahexamer contacts in the Gag lattice [24,50-52,66,67]. The globular CA_{CTD} domain (colored blue-green in Fig. 1-3) is composed of a short 3_{10} helix followed by an extended strand and four α-helices (CA helices 8-11) [68,69]. The strand/turn/helix 8 element is termed the major homology region (MHR) because its sequence is highly conserved across most retroviruses and retrotransposons [2,70]. Mutations in conserved MHR residues inhibit the assembly of immature particles, indicating that the MHR plays an important role in Gag lattice formation [47,50,70,71].

HIV-1 CA dimerizes in solution, and CA_{CTD} constructs have been crystallized as two distinct, but related dimers. One dimer is a side-by-side complex that is stabilized primarily by parallel hydrophobic packing of helix 9 against a symmetry mate (Fig. 2e) [68,69]. The RSV CA_{CTD} forms a similar dimer at low pH, albeit with a more hydrophilic interface and a somewhat different interdomain angle (R.L. Kingston, pers. comm.). The second HIV-1 CA_{CTD} dimer is a "domain-swapped" analog in which the MHR segments from each monomer associate to create a more extended dimer interface (Fig. 2f) [72]. In this case, the structure was determined for a CA_{CTD} mutant that lacked a single amino acid residue in the helix 8/9 linker, which favored the domain-swapped conformation. Mutations expected to disrupt intermolecular helix 9 packing interfaces seen in both types of dimer reduced Gag dimerization (and trimerization) *in vitro*, and inhibited the production of immature particles, supporting the biological relevance

of the interface(s) [42,43,47]. Possible roles for the two different CA_{CTD} dimers are discussed in greater detail below.

Residues located at the C-terminal end of CA and at the N-terminal end of the SP1 spacer are also essential for immature particle formation [48,73]. In the tomographic reconstruction of frozen-hydrated immature particles, density ascribed to the CA_{CTD}-SP1 region was located immediately beneath the central pore of each hexamer (i.e., the bottom of the cup), suggesting that this region stabilizes the hexamer. NMR studies of isolated Gag fragments indicate that the helical propensity for the CA-SP1 junction is weak [74], but sequence analyses and mutational studies support the idea that this region may adopt an ordered helical structure in assembled virions [73,75,76]. The CA-SP1 junction was therefore modelled as a six-helix bundle in the tomographic study (Fig. 2c and d). MLV Gag, which lacks a spacer peptide between its CA and NC domains, may contain an equivalent region at the C-terminus of the CA domain, termed the "charged assembly helix", which is also required for immature particle assembly [77].

Finally, the NC region of Gag (colored blue in Fig. 1b) forms the innermost layer of the immature virion and also plays essential roles in particle formation [21,23,51,78-81]. NC/RNA complex(es) do not follow the hexagonal symmetry of the CA and SP1 regions, and most experiments indicate that NC primarily "tethers" Gag molecules together, probably via RNA bridges, although additional NC-NC interactions may also occur [79,80,82,83]. NC/RNA tethers presumably increase the effective concentration of assembling Gag molecules, which could also play a more active role in orienting or otherwise facilitating CA-CA interactions.

While NC can bind RNA nonspecifically, packaging of the genome requires specific recognition of the dimeric, full-length viral RNA transcript. HIV-1 genome packaging requires an RNA element located near the 5′ end of the *gag* gene (termed "Ψ"), which is composed of four stem-loops that can dimerize through self-complementary base pairing interactions of loop 1 residues (Fig. 2g) (reviewed in [84]). Structures of NC in complex with two of the isolated stem loops from HIV-1 Ψ show that the CCHC zinc fingers of NC make sequence-specific, but remarkably different, contacts with exposed loop residues (Fig. 2h) [85,86]. The NC/RNA interactions visualized in these structures undoubtedly contribute to the specificity of genomic RNA packaging, although the full mechanism by which HIV-1 Gag packages dimeric RNA genomes has yet to be delineated. Even greater progress on the mechanism of genomic RNA packaging has been made for RSV and MLV, where complexes of NC with minimal Ψ RNA elements are now available [87,88]. In the MLV case, the high-affinity NC binding site is initially sequestered by base pairing in the monomeric form of the viral RNA, and then becomes exposed in the dimeric RNA, providing an elegant mechanism for coupling genome dimerization and encapsidation [87].

As is clear from the above discussion, structural studies of the immature virion are still at an early stage, and important future goals include: (1) determining high resolution structures of Gag in its soluble dimeric and trimeric conformations [43], (2) extending the resolution of models for the assembled hexagonal Gag lattice, and (3) understanding the mechanism by which the Gag lattice curves into a spherical assembly (e.g., through the inclusion of pentameric declinations and/or through irregular juxtapositions of hexagonal sheets). These are challenging problems, as retroviruses are irregular, yet highly organized assemblies, and progress will therefore continue to require the coordinated application of multiple different structural methods.

Mature retroviral capsids are fullerene structures

Following Gag processing, ~1,500 copies of the mature CA protein reassemble to form the mature capsid (Fig. 3) [89-91]. Retroviral capsids appear to adopt preferred shapes: cones (e.g., HIV), cylinders (e.g., MPMV), or "spheres" (e.g., MLV, RSV). However, recent tomographic analyses of mature HIV-1 and RSV particles have revealed significant capsid polymorphism, including amorphous shapes, multiple and nested capsids, and incompletely closed shells [92-94]. These observations suggest that mature retroviral capsids assemble through favored, but not strictly defined pathways.

The conical HIV-1 capsid is a curved hexagonal assembly called a fullerene cone [89-91,95] (Fig. 3b). Fullerene cones require twelve pentameric declinations ("pentons") to close and can adopt one of five allowed cone angles. The narrowest allowed cone angle (19.2°) predominates in both authentic HIV-1 capsids and in "synthetic" capsids assembled from pure recombinant proteins [89,93,96]. These narrow cones are closed by inclusion of five and seven pentons at their narrow and wide ends, respectively (Fig. 3b). The tubular and spherical shapes of other retroviral capsids can also be explained as related fullerene assemblies [89]. Specifically, spherical shapes are formed when the pentons are dispersed more evenly throughout a curved hexagonal lattice, and tubular capsids are formed by a cylindrical hexagonal lattice that is closed by inclusion of six pentons at either end of the tube [89,90,97,98].

Tomographic studies of frozen-hydrated mature HIV-1 particles support the fullerene organization of the viral capsid and reveal regions of high density immediately inside the broad end of the cone, which presumably represent sites of close contact with the genomic ribonucleoprotein complex [92,93]. The broad end of the capsid appears to reside at a constant (11-12 nm) distance from the matrix, indicating that the two layers may interact. The narrow end of the cone has been reported to approach the membrane even more closely than the broad end, and in some cases, appeared connected to the membrane/matrix, which may represent nucleation sites where capsid assembly is initiated [92]. Direct capsid/matrix linkages were also observed in a recent tomographic study of mature RSV particles [94], and the linkage sites frequently sat beneath Env spikes and linked to capsid vertices (i.e., pentons). This may imply that Env assemblies help initiate capsid assembly and/or promote formation of pentameric declinations, although it should be noted that specific Env linkages cannot be absolutely required for functional capsid formation, since retroviruses remain infectious when pseudotyped with heterologous envelope proteins.

Structure of the hexameric CA lattice

Pure recombinant HIV-1 CA proteins and CA-SP1-NC/RNA complexes can form cylindrical and conical shells *in vitro*, implying that all of the information necessary to form a conical capsid resides within the CA polypeptide [89,99-101]. Mature HIV-1 and RSV CA proteins form hexagonal arrays of hexameric CA_{NTD} rings, with each ring connected to its six nearest neighbors via CA_{CTD} dimer interactions [90,91,97]. A crystal structure of the N-MLV CA_{NTD} hexamer revealed that the subunits associate via an 18-helix bundle formed by helices 1-3 [61]. Recently, electron cryocrystallography of well-ordered two-dimensional crystals of full-length HIV-1 CA yielded a structure of the complete hexagonal lattice at 9 Å resolution [102]. At this resolution, it was possible to resolve rods of density that corresponded to all of the CA secondary structural elements, allowing unequivocal positioning of the CA_{NTD} and CA_{CTD} crystal structures. The resulting pseudoatomic model showed that the mature HIV-1 capsid lattice is stabilized by three different intermolecular CA-CA interfaces (Fig. 3c-e): (1) intrahexamer CA_{NTD}-CA_{NTD} interactions are mediated by CA helices 1-3, which associate as an 18-helix bundle in the center of the hexamer (as seen for the N-MLV CA_{NTD} hexamer), (2) intrahexamer CA_{NTD}-CA_{CTD} interactions are formed by asymmetric insertion of the CA_{NTD} helix 4 into a groove of CA_{CTD} from an adjacent molecule within the hexamer, allowing the

six CA_{CTD} domains to form a "belt" surrounding the CA_{NTD} core, and (3) interhexamer CA_{CTD}-CA_{CTD} interactions are mediated by symmetric, parallel dimerization of helix 9 from CA_{CTD} subunits of adjacent hexamers (as seen in the side-by-side CA_{CTD} dimer) (Fig. 2c) [68,69]. Gratifyingly, this pseudoatomic model of the hexagonal capsid lattice is consistent with a large body of biochemical and genetic data, including deuterium exchange, crosslinking, mutagenesis, and second-site suppressor studies (e.g., see Fig. 3c and d) [47,58,98,103,104].

Although a molecular model for the hexagonal CA lattice is now in hand, we still lack a comprehensive, high-resolution structural model of the mature HIV-1 capsid. Obtaining such a model will require: (1) extending studies of the hexameric CA lattice to atomic resolution, (2) determining how this lattice accommodates conical curvature, (3) obtaining a structural description of the pentameric declinations, (4) determining precisely how the ends of the capsid interact with the matrix shell, and (5) defining how the core components, particularly the replication-initiation complex and associated enzymes, are organized within the capsid.

Retroviral maturation

Gag processing by the viral protease (PR) initiates the essential process of virion maturation, in which the liberated NC/RNA complex condenses at the center of the core, the genomic RNA dimer becomes more stable [105], and the processed CA protein forms the conical capsid. HIV-1 PR and other viral enzymes encoded within Gag-Pol fusion proteins are generated when ~5% of the ribosomes shift to the -1 frame while translating the C-terminal end of *gag*, and the resulting Gag-Pol proteins are apparently packaged into virions via Gag-Gag lattice interactions. PR is liberated by autoproteolysis at a late stage of viral assembly, which ensures that Gag proteins are not processed before they assemble. All five different HIV-1 Gag processing sites are essential for infectivity, and the sites are processed at different rates, in the order: SP1/NC > MA/CA and SP2-p6 > NC-SP2 > CA-SP1 [106].

Retroviral capsids do not assemble via a direct, concerted condensation of the immature lattice, but rather are likely reassembled from a subset of free CA subunits, following specific nucleation events that help restrict the overall capsid assembly pathway [20,58,92-94]. CA processing must therefore destabilize the immature Gag lattice while promoting formation of the mature capsid lattice. Proteolytic processing appears to trigger conformational changes at both ends of the CA protein that favor capsid assembly. Processing at the N-terminal end of CA (the MA-CA junction in HIV-1 Gag) converts the first 13 CA residues from an extended conformation into a folded β-hairpin structure (colored yellow in Fig. 3) [53,66,107]. This structure is stabilized by formation of a partially buried salt bridge between a highly conserved aspartate residue within CA helix 3 and the newly processed N-terminus [66,108]. β-hairpin formation functions as a switch that favors mature CA hexamer formation, though its effects appear subtle. In the mature CA hexamer, the β-hairpin sits immediately above the interface created by CA helices 1-3 but does not make extensive intermolecular interactions [61,102]. Rather, β-hairpin formation appears to help position CA_{NTD} helices 1-3 to form the mature capsid hexamer (and may also help disrupt immature lattice interactions).

Proteolysis at the C-terminal end of CA (the CA-SP1 junction of HIV-1 Gag) also appears to trigger a second molecular switch that favors mature CA lattice formation. In support of this idea, removal of SP1 can cause recombinant HIV-1 Gag proteins to switch from immature-like spherical assemblies to mature-like cylinders *in vitro* [13]. As discussed above, the CA-SP1 region appears to stabilize the immature Gag hexamer, and proteolysis at the CA-SP1 junction therefore presumably destabilizes the Gag lattice. This switch may also involve large rearrangements in the orientation of the CA_{CTD} dimer. As noted above, CA_{CTD} has been crystallized as both side-by-side and domain-swapped, dimers (Fig. 2e and f). One attractive model is that the domain-swapped dimer exists within the immature virion and then converts

into a side-by-side dimer in the mature capsid [72,109]. This would allow the more extended domain-swapped dimer to stabilize the assembly-competent immature virion, whereas the smaller side-by-side dimer interface could contribute to the lability of the disassembly-competent mature capsid. In principle, interactions within the SP1-NC region could also contribute to formation of domain-swapped dimers, rationalizing how proteolytic processing at the CA-SP1-NC junctions could promote the conformational switch. Moreover, the constraints of having to switch between monomers (when Gag is first made), to domain-swapped dimers (in the immature virion), to side-by-side dimers (in the mature capsid), offer an appealing explanation for the extreme conservation of the MHR. Consistent with this idea, conserved MHR residues form extensive hydrophobic interactions and hydrogen bonding networks in all three structures [68,69,72,110]. While this speculative model requires rigorous testing, it is not unreasonable to expect that CA may undergo major conformational changes during maturation, given the dramatic conformational rearrangements seen in other well characterized viral maturation processes [111,112].

Novel maturation inhibitors block Gag processing and CA assembly

The clinical success of HIV-1 PR inhibitors validates maturation as an attractive therapeutic target, and non-protease maturation inhibitors have recently been identified. One class of maturation inhibitors is exemplified by 3-O-(3′,3′-dimethylsuccinyl)betulinic acid (also called PA-457) [113,114], which inhibits HIV-1 replication in tissue culture and animal model systems, and is currently in Phase 2 clinical trials [115]. PA-457 inhibits Gag processing at the CA-SP1 junction and appears to bind directly to this site because the inhibitor is incorporated into assembling virions and because mutations at the CA-SP1 junction induce drug resistance [115-118]. PA-457 does not bind free Gag, however, and therefore presumably recognizes SP1 in its assembled conformation. Cells treated with PA-457 release noninfectious virions with aberrant capsids that resemble those seen for viruses with mutations that inhibit CA-SP1 processing [115]. However, it appears that PA-457 does not completely block, but simply delays, proteolysis of the CA-SP1 junction [119], suggesting that Gag proteolysis and maturation must occur within a defined temporal window.

A second class of inhibitors binds the processed CA protein and inhibits capsid assembly. The founding members of this class are methylphenylurea compounds, such as CAP-1, which bind in a deep pocket in CA_{NTD}, formed at the junction of helices 1, 2, 4, and 7 [120,121]. Interestingly, this pocket is not present in the static structure of free CA_{NTD}, but rather is created when the conserved CA Phe32 residue swings out into solution. Another inhibitor of this type is CA-I, a 12-residue peptide identified by phage display, which binds between CA_{CTD} helices 8 and 11 [122,123]. Both CA-I and CAP-1 bind within (or adjacent to) the mature CA_{NTD}-CA_{CTD} interface (Fig. 3e), suggesting that they inhibit capsid assembly by disrupting this interaction [102,121,123]. CA-I and CAP-1 bind too weakly to be clinically useful, but the independent discovery of two different inhibitors that bind at the CA_{NTD}-CA_{CTD} interface makes this site an attractive target for further inhibitor development.

Capsid disassembly

The fate of the core particle in the cytoplasm of newly infected cells represents perhaps the most interesting, yet poorly understood stage of the retroviral life cycle. In essence, we still need to learn why retroviruses go to the trouble of building the mature core particle, and how host proteins can positively and negatively regulate its different functions. Following fusion of the viral and cell membranes, the HIV-1 core is released into the cytoplasm, the capsid uncoats, the single-stranded RNA genome is reverse-transcribed into double-stranded DNA, and the proviral DNA genome is trafficked into the nucleus and integrated into host chromosomes (Fig. 1a) (reviewed in refs [124-126]). The HIV-1 preintegration complex travels

to the nuclear envelope via the microtubule network [127], and is subsequently actively transported through the nuclear pore, although the *cis*- and *trans*-acting factors required for nuclear uptake are still debated [128]. Genetic analyses indicate that properly assembled capsids are required for the successful completion of reverse transcription (and other early events) [47,49,50,67,71,129,130] and also for later events that accompany nuclear localization of the preintegration complex [130,131]. Moreover, mutations that either increase or decrease capsid stability can reduce viral infectivity, suggesting that the timing (or extent) of capsid disassembly is probably important for successful completion of the first half of the viral life cycle [129].

Capsid restriction

One of the most exciting recent developments in retrovirology is the realization that cells possess a number of intrinsic antiviral responses that can potently inhibit, or restrict, viral replication (reviewed in [132]). One very important family of cellular restriction factors is the tripartite motif 5 (TRIM5) proteins, which mediate species-specific early blocks to retrovirus infection. TRIM5 proteins come in two distinct forms (Fig. 4a): TRIM5α (expressed by most primates) [133] and TRIM-Cyp (expressed by owl monkeys) [134] (see [135-137] for recent reviews). Both TRIM alleles recognize the viral capsid, but do so in different ways. TRIM5α restriction is species- and virus-specific, and the specificity of restriction is largely determined by interactions between the C-terminal SPRY/B30.2 domain and the intact retroviral capsid. Although structural details are still lacking, mutational studies and sequence analyses of different SPRY alleles have provided insights into the determinants of capsid recognition (e.g., see [138-141]). In TRIM-Cyp, the SPRY domain of TRIM5α is replaced by cyclophilin A (CypA), a well-known peptidyl prolyl isomerase. In this case, the mechanism of capsid recognition is better understood because CA/CypA interactions have been characterized extensively. CypA makes sequence-specific contacts with the extended loop that connects CA helices 4 and 5, burying the CA Pro90 sidechain in the enzyme's active site [141,142]. Pro90 is efficiently isomerized by CypA [143], although the biological relevance of this activity remains to be determined.

As illustrated in Figure 4c, the cyclophilin-binding loop sits on the surface of the capsid, on the outer edge of the CA hexamer. TRIM5 proteins are apparently trimeric, and it is therefore likely that they bind over one of the two sites of local three-fold symmetry on the capsid surface, most probably on the site between three adjacent hexamers (red in Fig. 4c) [145]. Although the subsequent events that lead to restriction are not yet well understood, TRIM5α and TRIM-Cyp typically prevent accumulation of reverse transcripts (although later-stage blocks are observed under some restricting conditions [146,147]). TRIM5α restriction correlates with accelerated rates of retroviral capsid dissociation, leading to the proposal that TRIM5α (and its cofactors) destabilize the capsid before it can perform essential functions [138,148]. Alternatively, TRIM5α may inactivate cores by diverting them to cytoplasmic "TRIM bodies" and/or to the proteasome (e.g., see ref. [147]). This is a very active area of research, and ongoing functional studies will likely soon set the stage for structural studies on the mechanism of TRIM5 restriction.

Virus assembly *in vitro* and *in vivo*

This review has summarized our growing understanding of the structures of immature and mature retroviral capsids. It is quite remarkable that accurate mimics of both structures can be assembled *in vitro* using pure recombinant proteins and non-specific nucleic acids. Indeed, these (and other viral systems) provide dramatic extensions of Anfinsen's original hypothesis by showing that primary polypeptide sequences can encode all of the information necessary to dictate the assembly of megadalton virus-like particles, *even when those particles lack any*

formal symmetry. Of course, eukaryotic cells contain elaborate pathways that are used by viruses to control the localization, timing, and efficiency of particle assembly, and the next frontier is to understand how retroviruses assemble in the complex environment of the cell.

Acknowledgments

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* of special interest

** of particular interest

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Abbreviations

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Env	envelope protein
CA$_{CTD}$	C-terminal domain of the CA protein
CA$_{NTD}$	N-terminal domain of the CA protein
CA-I	12-residue peptide that inhibits CA assembly
CAP-1	N-(3-chloro-4-methylphenyl)-N′-{2-[({5-[(dimethylamino)-methyl]-2-furyl}-methyl)-sulfanyl]ethyl}-urea), an inhibitor of CA assembly
CypA	cyclophilin A
Env	membrane envelope glycoprotein
Gag	the Gag polyprotein is the major structural protein of retroviruses
HIV	human immunodeficiency virus
MHR	major homology region
MLV	murine leukemia virus
M-PMV	Mason-Pfizer monkey virus
N-MLV	N-tropic murine leukemia virus
PA-457	3-O-(3′,3′-dimethylsuccinyl)betulinic acid, an inhibitor of capsid assembly
PR	retroviral protease
RING	really interesting new gene, a ubiquitin E3 ligase domain
RSV	Rous sarcoma virus
SP1	spacer peptide 1
SP2	spacer peptide 2
SPRY	conserved domain from the SPL A kinase and ryanodine receptor
TRIM5α	tripartite motif protein 5α
TRIM-Cyp	tripartite motif protein-cyclophilin A fusion
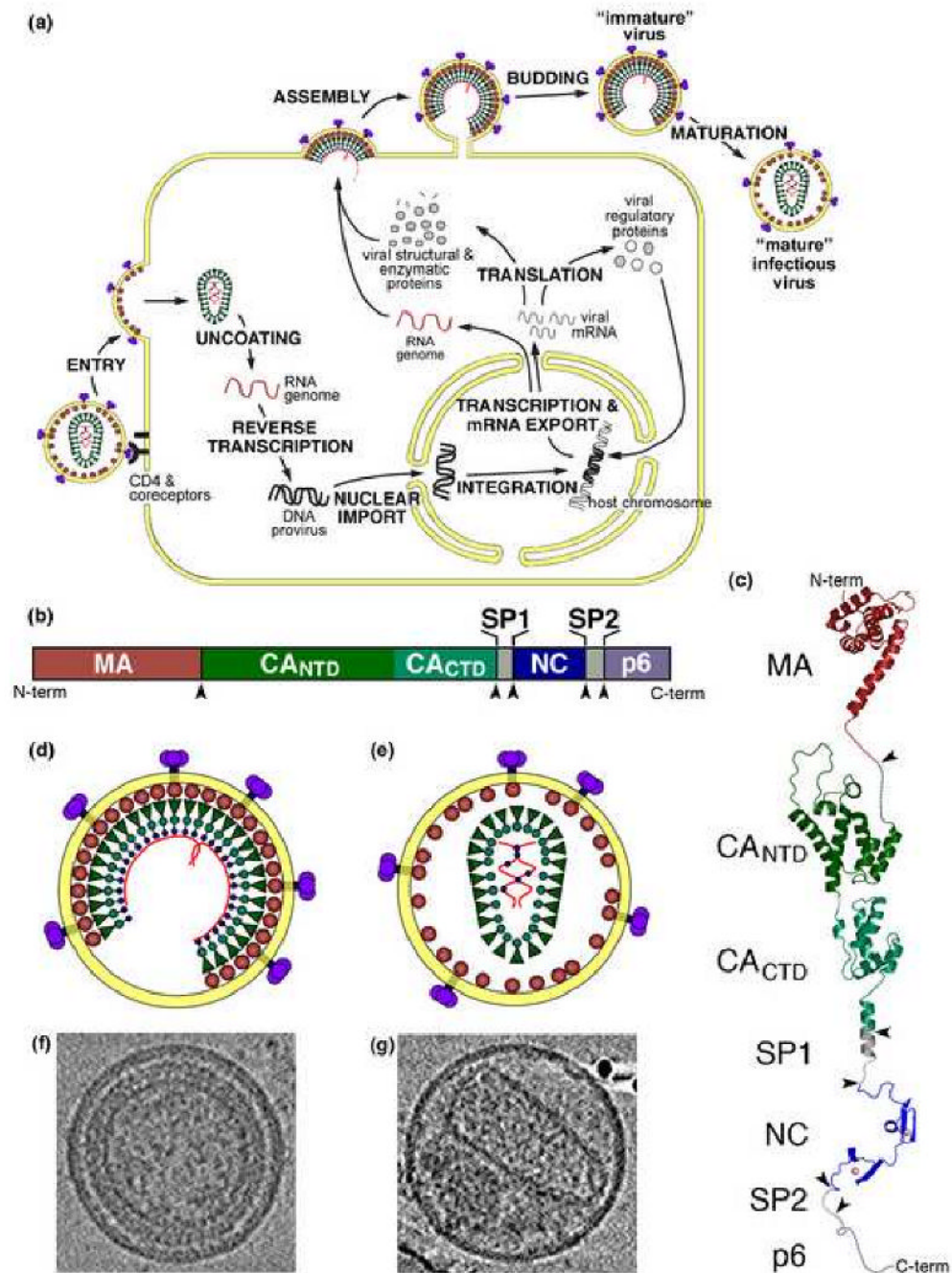
Figure 1.
(**a**) Summary of the HIV-1 replication cycle. (**b**) HIV-1 Gag polyprotein domain structure, showing the locations of MA, CA_{NTD}, CA_{CTD}, SP1, NC, SP2, and p6. (**c**) Structural model of the extended Gag polypeptide, derived from high-resolution structures and models of isolated domains. Unstructured and linker regions are represented by dashed lines. PR cleavage sites are indicated by the arrowheads in (**b**) and (**c**). (**d, e**) Schematic models of the immature (**d**) and mature (**e**) HIV-1 virions. (**f, g**) Central slices through cryo-EM tomograms of immature (**f**) and mature (**g**) HIV-1 particles. The spherical virions are approximately 130 nm in diameter.
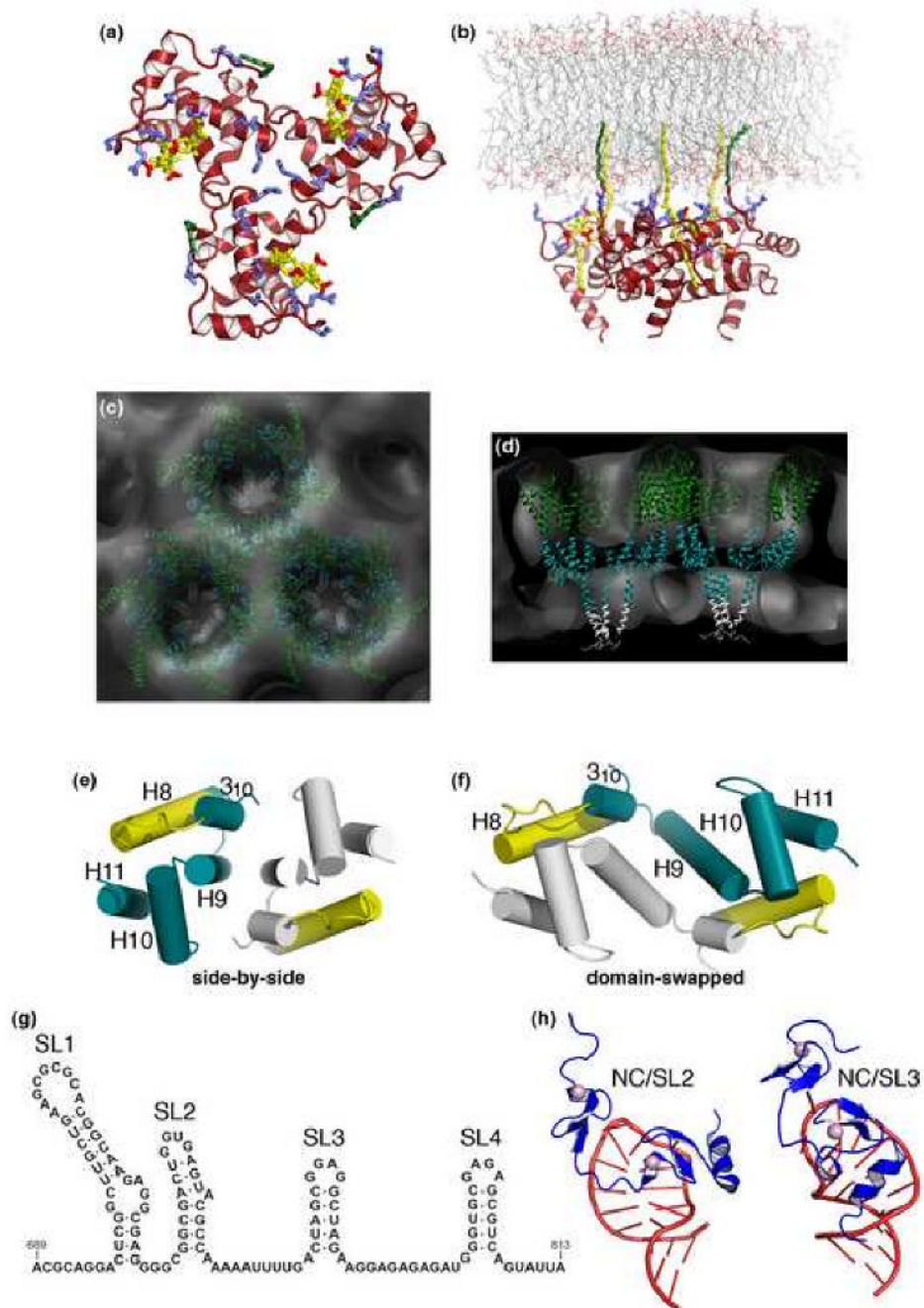
Figure 2. Structural features of the immature Gag lattice

(**a, b**) Models for membrane binding by the N-terminal MA domain showing a top view (**a**) and a side view of the MA trimer bound to a lipid bilayer (**b**), with the myristoyl chain (colored in green) inserted into the inner leaflet, and basic residues (blue) interacting with acidic phospholipid headgroups, including PI(4,5)P (yellow) [25,40]. (**c, d**) Two views of a model based on electron cryotomography of the immature Gag lattice (gray) showing that Gag-Gag lattice interactions are mediated primarily by the CA and SP1 domains. Plausible positions of the CA_{NTD} (dark green), CA_{CTD} (blue green), and SP1 (gray) layers are shown for reference [24]. (**e, f**) Two crystallographically characterized HIV-1 CA_{CTD} dimers showing side-by-side (**e**) [68,69] and domain-swapped conformations (**f**) [72]. The monomers are shown in blue

green and gray, and the MHR element in yellow. (**g, h**) Secondary structure of the HIV-1 Ψ packaging element (**g**), and high-resolution structures (**h**) of the isolated NC domain bound to the second (left) and third (right) Ψ stem loops, with NC in blue and RNA in red [85,86].
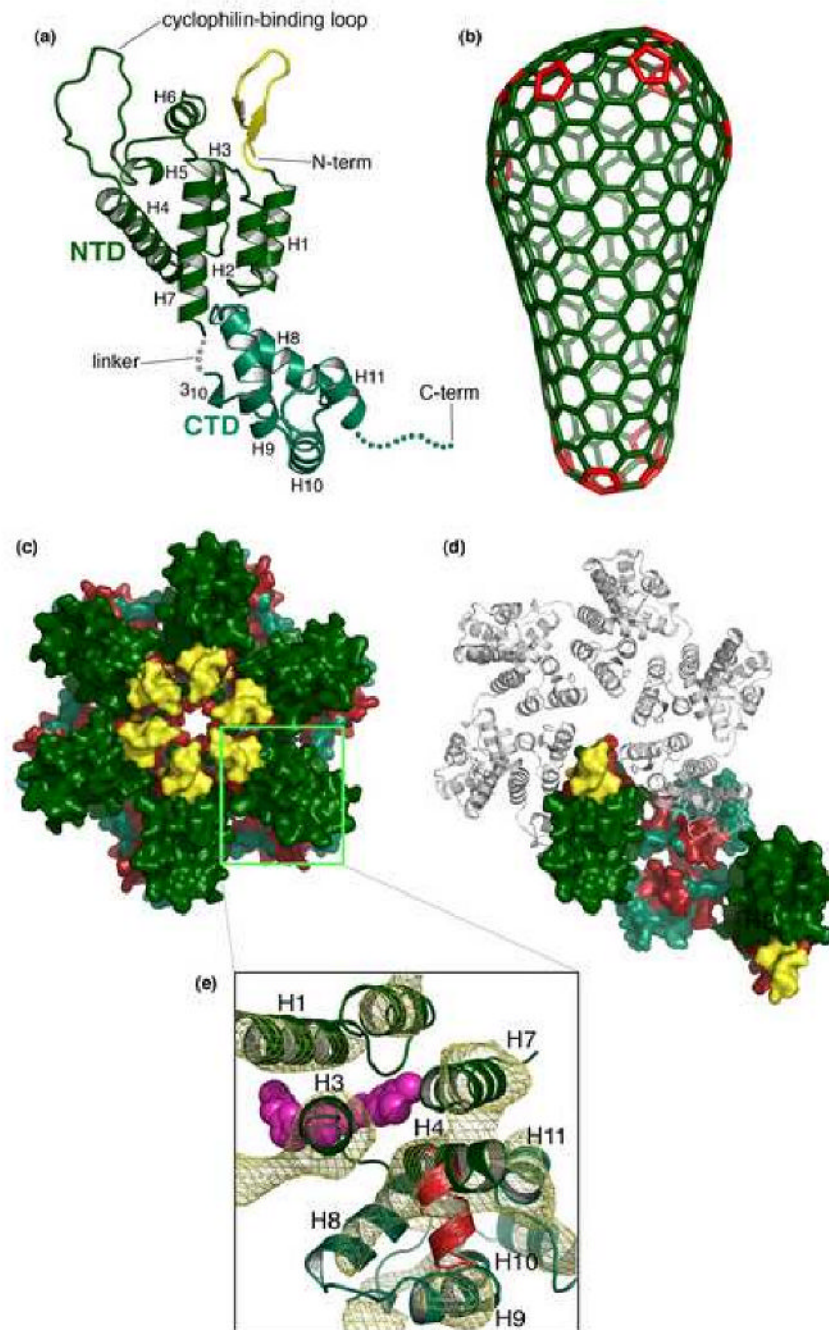
Figure 3. Structure of the mature HIV-1 capsid
(**a**) Tertiary structure of the mature, processed CA protein, with the N-terminal domain colored in dark green and the C-terminal domain in blue-green. The β-hairpin is colored yellow. The final 11 residues of CA, indicated by the dashed line are typically disordered in crystal structures. (**b**) Fullerene model for the conical capsid, with CA hexamers (green) and pentameric declinations (red) [89]. (**c, d**) Surface representation of the pseudoatomic model of the mature HIV-1 CA hexamer [102], emphasizing the CA_{NTD} hexamer (**c**) and CA_{CTD} dimer (**d**), viewed from the outer surface of the capsid. Color coding is the same as in (**a**), except with CA residues colored red to denote sites of significant protection from deuterium exchange in the hexagonal CA lattice [103]. Note that the exposed red patch in (**d**) corresponds to the

CA_{NTD}-binding site on the CA_{CTD}, which is exposed when the neighboring CA_{CTD} domain is removed. (**e**) Top view of the CA_{CTD}-CA_{NTD} intermolecular interface. The pseudoatomic model is shown in ribbon representation, fitted to the experimental density map derived by electron cryocrystallography (gold mesh) contoured at 1.6σ [102]. Binding sites of the CA-I (red) [123] and CAP-1 (magenta) [121] maturation inhibitors are indicated.
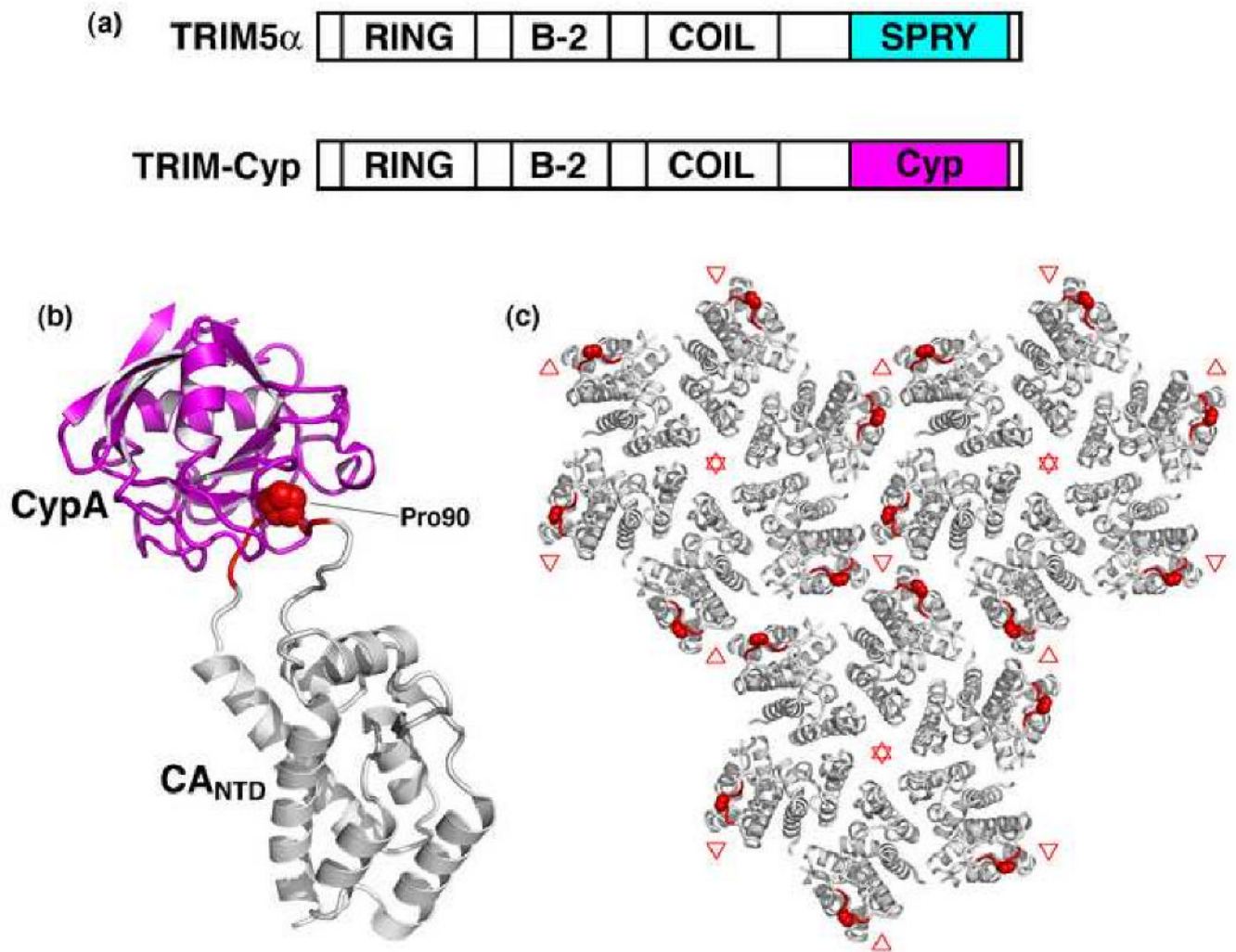
Figure 4. TRIM restriction factors
(**a**) Domain structures of TRIM5α and TRIM-Cyp highlighting the differences in their C-terminal capsid binding domains. (**b**) Structure of cyclophilin A (magenta) bound to CA$_{NTD}$ (gray) [142]. Cyclophilin-binding residues are colored red, with CA Pro90 shown explicitly. (**c**) Top view of the mature lattice, with three full hexamers [102]. Note that the cyclophilin-binding loops (red) surround local three-fold symmetry axes (denoted by red triangles).