HIV-1 Assembly, Budding, and Maturation

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A defining property of retroviruses is their ability to assemble into particles that can leave producer cells and spread infection to susceptible cells and hosts. Virion morphogenesis can be divided into three stages: *assembly*, wherein the virion is created and essential components are packaged; *budding*, wherein the virion crosses the plasma membrane and obtains its lipid envelope; and *maturation*, wherein the virion changes structure and becomes infectious. All of these stages are coordinated by the Gag polyprotein and its proteolytic maturation products, which function as the major structural proteins of the virus. Here, we review our current understanding of the mechanisms of HIV-1 assembly, budding, and maturation, starting with a general overview and then providing detailed descriptions of each of the different stages of virion morphogenesis.

The assembling virion packages all of the components required for infectivity. These include two copies of the positive sense genomic viral RNA, cellular $tRNA^{Lys,3}$ molecules to prime cDNA synthesis, the viral envelope (Env) protein, the Gag polyprotein, and the three viral enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN). The viral enzymes are packaged as domains within the Gag-Pro-Pol polyprotein, which is generated when translating ribosomes shift into the −1 reading frame at a site near the 3′ end of the *gag* open reading frame, and then go on to translate the *pol* gene.

HIV-1 virion assembly occurs at the plasma membrane, within specialized membrane microdomains. The HIV-1 Gag (and Gag-Pro-Pol) polyprotein itself mediates all of the essential events in virion assembly, including binding the plasma membrane, making the protein–protein interactions necessary to create spherical particles, concentrating the viral Env protein, and packaging the genomic RNA via direct interactions with the RNA packaging sequence (termed Ψ). These events all appear to occur simultaneously at the plasma membrane, where conformational change(s) within Gag couples membrane binding, virion assembly, and RNA packaging. Although Gag itself can bind membranes and assemble into spherical particles, the budding event that releases the virion from the plasma membrane is mediated by the host ESCRT (endosomal sorting complexes required for transport) machinery.

Folded domains within Gag are separated by flexible linker regions which contain the PR

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cleavage sites (Fig. 1B,C). The amino-terminal Gag domain is called MA, and it functions to bind the plasma membrane and to recruit the viral Env protein. The central domain of Gag is called CA, and it mediates the protein–protein interactions required for immature virion assembly and then creates the conical shell (called the capsid) of the mature viral core. The basic Gag NC domain contains two copies of the retroviral zinc finger motif. NC captures the viral genome during assembly, and also functions as a nucleic acid "chaperone" during $tRNA^{Lys,3}$ primer annealing and reverse transcription. Finally, the carboxy-terminal Gag p6 region contains binding sites for several other proteins, including the accessory viral protein Vpr, as well as two short sequence motifs, termed "late assembly domains," which bind the TSG101 and ALIX proteins of the cellular ESCRT pathway. Gag also contains two spacer peptides, termed SP1 and SP2, which help to regulate the conformational changes that accompany viral maturation.

The virion acquires its lipid envelope and Env protein spikes as it buds from the plasma membrane. Unlike Gag, Env is an integral membrane protein. It is inserted cotranslationally into ER membranes and then travels through the cellular secretory pathway where it is glycosylated, assembled into trimeric complexes, processed into the trans-membrane (TM; gp41) and surface (SU; gp120) subunits by the cellular protease furin, and delivered to the plasma membrane via vesicular transport.

The Gag polyprotein initially assembles into spherical immature particles, in which the membrane-bound Gag molecules project radially toward the virion interior (Fig. 1D,F). As the immature virion buds, PR is activated and cleaves Gag into its constituent MA, CA, NC, and p6 proteins, thereby also releasing the SP1 and SP2 peptides. Proteolysis is required for conversion of the immature virion into its mature infectious form (Fig. 1E,G). Like other retroviral proteases, HIV-1 PR is a dimeric aspartic protease (Fig. 1H). PR recognizes specific sites within Gag and cleaves them in an ordered fashion (Fig. 1B,C, arrowheads). Gag proteolysis triggers major changes which include condensing and stabilizing the dimeric RNA genome, assembling the conical capsid about the genomic RNA–NC-enzyme complex, and preparing the virion to enter, replicate, and uncoat in the next host cell. Thus, viral maturation can be viewed as the switch that converts the virion from a particle that can assemble and bud from a producer cell into a particle that can enter and replicate in a new host cell. The following sections review our current understanding of HIV-1 assembly, budding, and maturation.

VIRION COMPOSITION AND RNA PACKAGING

The main constituents of HIV-1 are Gag, which makes up ~50% of the entire virion mass and the viral membrane lipids, which account for ~30% of virion mass (reviewed in Carlson 2008). Other viral and cellular proteins together contribute an additional ~20%, whereas the genomic RNA and other small RNAs amount to ~2.5% of virion mass. Gag, Gag-Pro-Pol, Env, the two copies of genomic RNA, the tRNA primer, and the lipid envelope are all essential for viral replication, whereas the relevance of virion incorporation of other cellular and viral accessory proteins, small RNA molecules, and specific lipids is generally less well understood.

All viral gene products are encoded on the genomic RNA, which also serves as mRNA for Gag and Gag-Pro-Pol, whereas singly or multiply spliced RNAs are translated to produce Env and accessory proteins, respectively. Unspliced and incompletely spliced HIV-1 RNAs are exported from the nucleus via a Rev-dependent export pathway, whereas completely spliced mRNAs exit the nucleus via the normal mRNA export route. Translation of Gag, Gag-Pro-Pol and most accessory proteins occurs on cytosolic polysomes. The two viral membrane proteins, Env and the accessory protein Vpu, which are encoded by the same mRNA, are translated on the rough ER. All virion components need to traffic from their point of synthesis to sites of assembly on the plasma membrane. The coordinated synthesis of structural proteins and enzymes as domains of the Gag and Gag-

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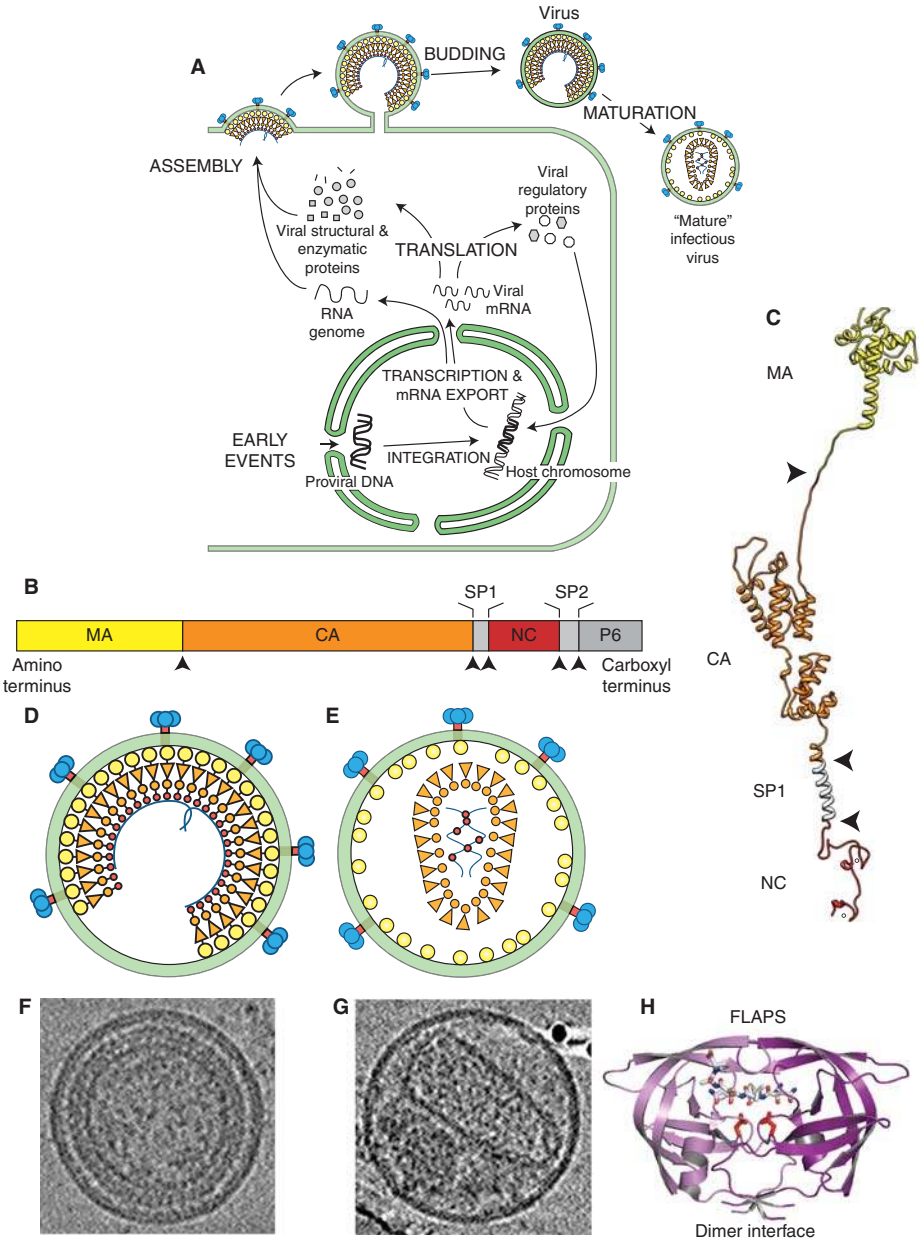

Figure 1. HIV-1 assembly, budding, and maturation. (*A*) Schematic illustration showing the different stages of HIV-1 assembly, budding, and maturation. (*B*) Domain structure of the HIV-1 Gag protein; arrows denote the five sites that are cleaved by the viral PR during maturation. (*C*) Structural model of the HIV-1 Gag protein, created by combining structures of the isolated MA-CA_{NTD} (2GOL), CA_{CTD} (1BAJ), and NC (1MFS) proteins, with a helical model for SP1. (*D*) Schematic model showing the organization of the immature HIV-1 virion. (*E*) Schematic model showing the organization of the mature HIV-1 virion. (*F*) Central section from a cryo-EM tomographic reconstruction of an immature HIV-1 virion. (*G*) Central section from a tomographic reconstruction of a mature HIV-1 virion. (*H*) Structure of HIV-1 protease (PR, 3D3T). The two subunits in the dimer are shown in different shades of purple, the "flap" and dimerization interfaces are labeled, positions of the active site Asp25 residues are shown in red, and a bound peptide corresponding to the SP2-p6 cleavage site is shown as a stick model, with oxygen atoms in red and nitrogen atoms in blue.

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Pro-Pol polyproteins (which are produced at a ratio of ~20:1) ensures that these components are made at the proper stoichiometry (Jacks et al. 1988). Gag then binds other virion components through direct protein–protein and protein–RNA interactions, which allows the virus to assemble all of its components using a single targeting signal (Frankel and Young 1998; Freed 2001).

Protein Trafficking and Virion Incorporation

The HIV Gag and Gag-Pro-Pol polyproteins traffic from their sites of synthesis in the cytoplasm to the plasma membrane and then sort into detergent-resistant membrane microdomains (Ono and Freed 2001). Virus production is cholesterol and sphingolipid dependent, and the virus is enriched in "raft"-associated proteins and lipids (Ono 2009). Gag has been reported to interact with the cellular motor protein KIF4 (Tang et al. 1999; Martinez et al. 2008) and with various components of intracellular vesicle trafficking pathways (Batonick et al. 2005; Dong et al. 2005; Camus et al. 2007), but the role of microtubules and/or membrane trafficking for Gag membrane transport remains unconfirmed. Gag molecules do not polymerize extensively before they reach the membrane (Kutluay and Bieniasz 2010). Instead, soluble monomeric Gag proteins appear to fold into a compact, auto-inhibited conformation(s), which subsequently undergoes conformational changes that cooperatively couple MA–membrane (see online Movie 1 at www.perspectivesinmedicine.org), NC–RNA, and Gag–Gag interactions (Chukkapalli et al. 2010; Datta et al. 2011a; Jones et al. 2011). Gag molecules thus arrive at the plasma membrane as small oligomers, probably monomers or dimers, which polymerize onto nucleation sites composed of Gag–RNA complexes (Jouvenet et al. 2009). The cellular ATPase, ABCE1, has also been implicated in binding and chaperoning membrane-bound assembly intermediates (Dooher et al. 2007), although mechanistic details remain to be elucidated.

Gag membrane targeting requires myristoylation and a basic patch on the MA domain as well as the plasma membrane-specific lipid phosphatidyl inositol (4,5) bisphosphate ($PI(4,5)P_2$ [Ono et al. 2004]). Binding of the MA^{Gag} domain to $PI(4,5)P_2$ exposes the amino-terminal myristoyl group (Saad et al. 2006, 2007), and this "myristoyl switch" provides an elegant mechanism for anchoring Gag stably on the inner leaflet (Movie 1; Fig. 2). Electrostatic interactions with acidic phospholipids, which are strongly enriched in the HIV-1 lipidome (Brugger et al. 2006), probably also contribute to membrane anchoring. MA domains from other retroviruses can also bind $PI(4,5)P_2$ (Hamard-Peron et al. 2010), but the energetics of $PI(4,5)P_2$ binding and myristoyl

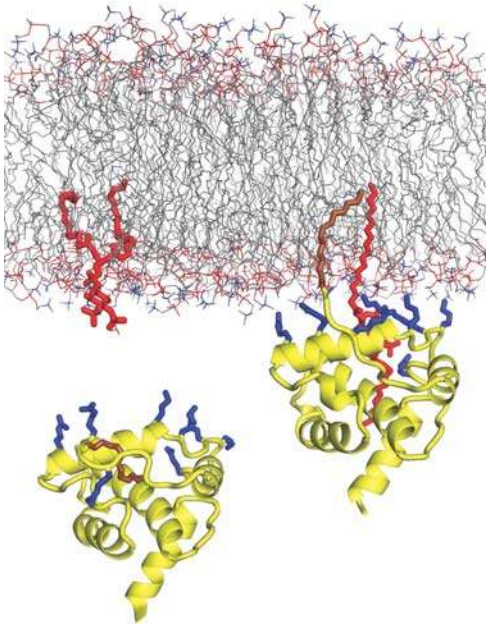

Figure 2. Myristoyl switch model for MA^{Gag} recognition of the plasma membrane (Saad et al. 2006). MA^{Gag} (yellow) proteins are shown with the aliphatic myristoyl group (brown) sequestered within the soluble protein (*left*, 1UPH), and with the myristoyl group extruded into the membrane when bound to the plasma membrane specific phosphatidyl inositide, $PI(4,5)P_2$, shown in red (*right*, 2H3F). The $PI(4,5)P_2$ inositol head group and unsaturated 2′-fatty acid bind within MA, allosterically inducing extrusion of the myristoyl group, whereas the saturated 1′-fatty acid of $PI(4,5)P_2$ remains embedded in the membrane. Basic residues on the membrane binding surface of MA^{Gag} are shown in blue.

sequestration vary, and not all retroviral MA proteins have amino-terminal myristoyl groups, suggesting that additional factors may govern membrane targeting, at least in those cases (Saad et al. 2008; Inlora et al. 2011).

The viral Env glycoproteins reach the plasma membrane independently of Gag. Genetic and biochemical analyses indicate that the long intracellular tail of TM helps sort Env into "raft"-like domains and mediates specific interactions with MA^{Gag} that promote Env virion incorporation (Yu et al. 1993; Cosson 1996; Murakami and Freed 2000; Wyma et al. 2000). Deletion of the TM cytoplasmic tail abolishes viral infectivity in most cell lines, but does not prevent Env incorporation (Einfeld 1996). Moreover, virions can be efficiently pseudotyped by heterologous glycoproteins without specific HIV-1 Gag interactions (Briggs et al. 2003a), implying that the MA–Env interaction is not absolutely essential for Env incorporation. Interestingly, pseudotyped particles seem to segregate into distinct classes, apparently displaying only one or the other glycoprotein (Leung et al. 2008). HIV-1 displays only ~7–14 glycoprotein trimers per virion (Chertova et al. 2002; Zhu et al. 2006). This number is considerably lower than for the related simian immunodeficiency virus, which has approximately 80 Env trimers per virion (Zhu et al. 2006), and suggests that clustering of the sparsely distributed Env trimers may be important for HIV-1 entry (Sougrat et al. 2007). Other constituents of the virion are the accessory protein Vpr (incorporated at a ratio of 1:7 to Gag [Muller et al. 2000]) through a specific interaction with the p6 domain (Kondo et al. 1995) as well as a few copies of the accessory proteins Vif and Nef.

Analysis of purified virion preparations by immunoblotting or mass spectroscopy has identified many cellular proteins in HIV-1 particles (Ott 2008), but their importance for virus assembly, budding, and/or infectivity is currently not well established in most cases. These cellular proteins include plasma membrane proteins like ICAM-1 (which may mediate virus adherence to cells) and HLA-II (which may modulate immune responses), as well as cytoplasmic proteins which may be incorporated via direct or indirect Gag interactions (e.g., actin and actin-binding proteins, cyclophilin A, ubiquitin, lysyl-tRNA-synthetase, and many RNA-binding proteins [Ott 2008]).

Viral Lipid Composition

HIV-1 buds at the plasma membrane of infected cells and the viral membrane is therefore derived from the cellular plasma membrane. Aloia et al. (1988, 1993) initially reported differences between the lipid compositions of the producer cell and HIV-1 membranes, with virion enrichment of sphingomyelin (SM), phosphatidyl serine (PS), phosphatidyl ethanol (PE), and cholesterol, as well as decreased membrane fluidity. Recent advances in lipid mass spectrometry have allowed a comprehensive, quantitative analysis of the entire lipid composition (the lipidome) of purified HIV-1 including determination of side chains. These analyses revealed strong virion enrichment of the "raft lipids" SM, cholesterol, and plasmalogen-PE, with an increase in saturated fatty acids compared with the producer cell membrane. The inner leaflet of the viral membrane is enriched in PS, and the overall lipid composition of HIV-1 strongly resembles detergent-resistant membranes isolated from producer cells (Brugger et al. 2006). The native HIV-1 membrane exhibits a liquid-ordered structure (Lorizate et al. 2009), providing further evidence for its raft-like nature. The HIV-1 membrane is also enriched in $PI(4,5)P_2$, consistent with the idea that this phosphatidylinositide plays an important role in targeting Gag to the membrane (Chan et al. 2008; also see above). Membranes of the murine leukemia virus are also enriched in cholesterol, ceramide, and glycosphingolipids (Chan et al. 2008), indicating that other retroviruses also bud from raft-like membrane domains.

RNA Trafficking and Incorporation

HIV-1, like all retroviruses, selectively incorporates two copies of the capped and polyadenylated full-length RNA genome into the virion

(Johnson and Telesnitsky 2010; Lever 2007). The two RNA strands are noncovalently dimerized in their 5′UTR. RNA dimerization initiates through formation of a "kissing-loop" structure mediated by Watson–Crick base pairing of the self-complementary sequence within the loop of the dimer initiation site (DIS) (Fig. 3), which then expands into a more extended helix linkage during viral maturation. RNA dimerization is required for RNA packaging and viral infectivity (Moore and Hu 2009), and involves *cis*-acting sequences within the 5′UTR of the viral genome, which are recognized by the NC^{Gag} domain. RNA–Gag interactions also appear to convert the compact, auto-inhibited Gag conformation into its extended assembly conformation which is required for virus assembly (Rein et al. 2011). Removal of the RNA packaging signal does not abolish particle production, but the resulting particles do contain abnormally high levels of nonspecific cellular mRNAs (Rulli et al. 2007), implying that RNA facilitates virion assembly by concentrating and aligning Gag molecules at the plasma membrane.

Although NC can bind RNA nonspecifically, genome packaging requires specific recognition of the dimeric, unspliced HIV-1 RNA. Recent single virion analyses by fluorescence microscopy have confirmed that nearly all HIV-1 particles contain genomic RNA, which is dimeric in most cases (Chen et al. 2009). Efficient genome packaging depends on a structural element of approximately 150 nucleotides located in the 5′ region spanning the major splice donor and the Gag initiation codon (the Ψ-site) (D'Souza and Summers 2005). The requirements for elements located downstream from the first splice donor explain why unspliced viral RNA is selectively packaged, at least in the case of HIV-1. The Ψ-site comprises four stable stem loop structures within the highly structured 5′UTR (Fig. 3), but efficient genome packaging appears to be affected by almost the entire 5′UTR, particularly elements close to the DIS. Structural analyses of individual loops from the Ψ-site in complex with NC revealed specific interactions between the CCHC-type zinc knuckles of NC and exposed loop residues of the RNA (Fig. 3), providing insight into the mechanism of RNA recognition (see online Movie 2 at www.perspectivesinmedicine.org). The structural basis of dimeric RNA packaging is best understood for the genome of murine leukemia virus, where the high-affinity NC binding sites are sequestered and become exposed only upon RNA dimerization

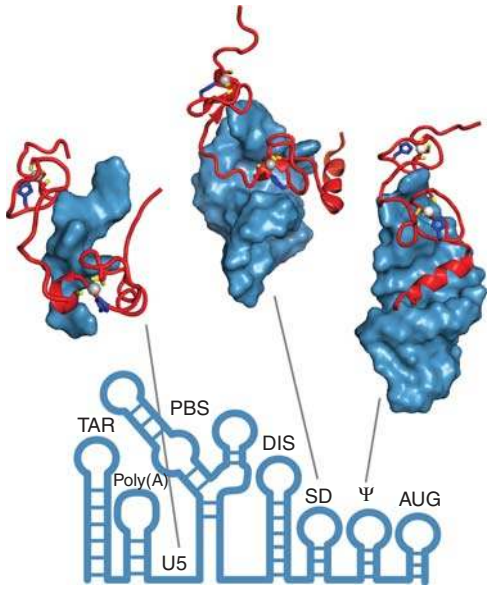

Figure 3. 5′ Untranslated region (UTR) of the HIV-1 RNA genome and its interactions with the viral NC protein. *Lower* image shows a secondary structure model for the 5′UTR, highlighting the TAR stem loop structure (which binds the viral Tat protein), the polyadenylation site, the U5 element, the primer binding site (PBS, which anneals to the $tRNA^{Lys,3}$ primer), and four stem loops within the packaging site, which contain the dimer initiation site (DIS, stem-loop I, which forms a kissing loop structure that initiates association of the two copies of the genomic RNA), the splice donor (SD, stem loop II, which acts as the 5′ donor for splicing of subgenomic RNAs), the Psi site (ψ, stem loop III, which forms an essential part of the packaging signal), and the Gag start codon (AUG, stem loop IV, which contains the start site for Gag translation). *Upper* structures show three different complexes between the NC protein (red, with zinc atoms shown in grey and Zn-coordinating side chains shown explicitly) and viral RNAs (blue), corresponding to the U5 region (Spriggs et al. 2008), the SD stem loop (1F6U), and the ψ stem loop (1A1T).

(Miyazaki et al. 2010). Retroviruses can copy information from either of their two packaged RNA strands during reverse transcription. This "pseudodiploid" property confers the distinctive advantage of high recombination potential and helps retroviruses to overcome environmental and therapeutic pressures.

Gag polyproteins of avian and murine retroviruses and of foamy virus reportedly enter the nucleus, where they bind newly transcribed RNA and cotraffic to sites of assembly (Schliephake and Rethwilm 1994; Scheifele et al. 2002; Andrawiss et al. 2003). Nuclear import and export has also been suggested for HIV-1 Gag (Dupont et al. 1999), but productive Gag-RNA packaging interactions appear to occur in the cytoplasm in this case. A cell fusion–dependent recombination assay was used to show that HIV-1 RNA dimerization, which is essential for packaging, occurs in the cytoplasm (Moore et al. 2009), and that dimerization frequency depends on the complementarity of the DIS loop sequences (Fig. 3). Overall, current data support a model in which genomic RNA dimerizes and associates with a few Gag molecules in the cytoplasm. These RNP complexes then traffic to the plasma membrane where they nucleate assembly (Kutluay and Bieniasz 2010).

In addition to the viral genome, HIV-1 particles also package small cellular RNAs, most notably tRNAs required for the initiation of reverse transcription (Kleiman et al. 2010). Two copies of $tRNA^{Lys3}$ anneal via Watson–Crick base pairing to an 18 base-pair sequence known as the primer binding sites (PBS), which is located within the 5′LTR (Fig. 3). The $tRNA^{Lys1,2}$ isoacceptors are also selectively packaged, and it has been suggested that Gag-Pro-Pol, genomic RNA, and lysyl-tRNA synthetase are all involved in specific tRNA packaging (Kleiman et al. 2010). HIV-1 particles also contain 7SL, the RNA component of host signal recognition particle (SRP), at a sevenfold molar excess over genomic RNA (Onafuwa-Nuga et al. 2006). However, SRP protein constituents are not incorporated and the functional significance of 7SL incorporation is unknown. Other RNA constituents of the virion include the 5S, 18S, and 28S rRNAs, which may simply be present owing to their cytoplasmic abundance.

HIV ASSEMBLY AND THE IMMATURE LATTICE

Direct visualization of individual budding and release events at high temporal resolution using fluorescently labeled Gag polyproteins and live-cell microscopy has recently revealed the kinetics of HIV-1 assembly and host cell factor recruitment (Jouvenet et al. 2008; Ivanchenko et al. 2009). Furthermore, live-cell fluorescence imaging revealed cytoplasmic genomic RNA to be highly dynamic in the absence of Gag, whereas a fraction of RNA molecules became immobilized at specific sites at the plasma membrane upon coexpression of Gag (Jouvenet et al. 2009). These sites marked the position of subsequent assembly events in most cases. Gag levels increase exponentially at individual plasma membrane assembly sites until a plateau is reached and budding ensues. The mean assembly time for individual particles is ~10 min, albeit with significant variability between sites (Jouvenet et al. 2008; Ivanchenko et al. 2009). Assembling Gag molecules are largely derived from the rapidly diffusing cytoplasmic pool (Ivanchenko et al. 2009) and do not enter via lateral diffusion within the membrane or from vesicle-associated Gag transport.

Gag assembly leads to formation of the immature lattice. Immature particles have historically been studied using virions that lacked PR activity or using virus-like particles assembled in vitro from bacterially expressed Gag proteins. However, recent direct imaging of budding viruses has confirmed that the immature Gag lattice is established as the virus assembles (Fig. 4A; Carlson et al. 2010). The Gag molecules in the immature virion are extended and oriented radially, with their amino-terminal MA domains bound to the inner membrane leaflet and their carboxy-terminal p6 domains facing the interior of the particle (Fig. 4B; Fuller et al. 1997). The immature lattice is stabilized primarily by lateral protein–protein interactions involving the CA-SP1 region, with the carboxy-terminal domain of

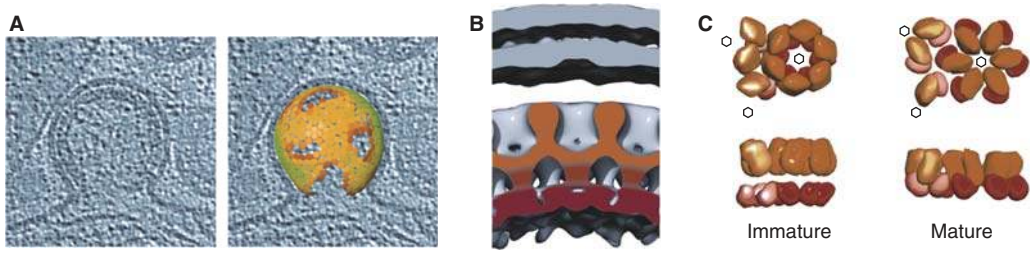

Figure 4. Assembly and structure of immature HIV-1 particles. (*A*) (*Left*) Central slice through a cryo-EM tomogram of a budding HIV-1 virion. (*Right*) Map of the Gag lattice in the budding virion. Positions of Gag hexagons are colored according to their hexagonal order, from low (brown) to high (green). (*B*) Cryo-EM tomogram of an immature HIV-1 virion, showing the structure of the immature HIV-1 Gag lattice. The surface was cut perpendicular to the membrane to reveal the two membrane leaflets, the two CA domains (orange and burnt orange), and the NC layer (red). (*C*) Schematic of the conformational rearrangements in the capsid lattice during maturation. (*Left*) Arrangement of the amino-terminal (orange) and carboxy-terminal (burnt orange) domains of CA in the immature lattice, viewed from outside the particle (*upper*), and rotated 90° around the horizontal axis (*lower*). Domains from neighboring hexamers are indicated in lighter colors, and sixfold lattice positions are marked by hexagons. (*Right*) Equivalent interactions of CA subunits within the mature HIV-1 capsid lattice.

CA (CA_{CTD}) and SP1 making particularly important interactions (Fig. 4B). MA and NC help to align and concentrate Gag molecules, but MA and p6 are dispensable for immature lattice formation, and NC can be replaced by a heterologous protein dimerization domain (Zhang et al. 1998; Accola et al. 2000). Extensive mutational analyses have identified the contributions of individual residues to immature and mature particle assembly (e.g., von Schwedler et al. 2003). These studies have generally confirmed the critical importance of the CA_{CTD} and SP1 regions for immature particle assembly, and in particular of the "major homology region" within CA_{CTD} which is conserved across retroviruses.

Over the past few years, cryoelectron tomography and image processing analyses have defined the three-dimensional structure of the immature Gag lattice in greater detail (Wright et al. 2007; Briggs et al. 2009). These studies confirmed that Gag molecules are arranged as hexamers with 8 nm spacings (Fig. 4). The CA_{NTD} forms six-membered rings with large central holes (Fig. 4B). At the current resolution (~2 nm), high-resolution structures cannot be unequivocally positioned within the density maps, but the lattice does not appear to be consistent with hexameric arrangements seen in crystallographic studies of the murine leukemia virus CA_{NTD} (Mortuza et al. 2004). Density for the CA_{CTD} domain resides beneath the hexamers, and the domain appears to make both intra- and interhexameric contacts (Fig. 4B and C). Like the full-length CA protein, CA_{CTD} constructs dimerize in solution and several different high-resolution CA_{CTD} structures have been reported, two of which appear to be used in constructing the mature capsid lattice (see below). Two other CA_{CTD} dimer structures have been suggested to play a role in stabilizing the immature lattice: a domain-swapped dimer (Ivanov et al. 2007) in which the MHR elements from each monomer associate to create a large interface, and a CA_{CTD} dimer observed in the presence of a peptide assembly inhibitor (CAI) or for certain mutations in the CAI binding pocket (Ternois et al. 2005; Bartonova et al. 2008). The CAI-induced dimer appeared to be the best fit to the reconstructed EM density, but it is currently unclear whether any of the CA_{CTD} dimer structures is actually reconstituted within the immature lattice, and higher resolution structures of immature particles are eagerly awaited.

Residues at the carboxy-terminal end of CA and in the adjacent SP1 region are also essential for immature lattice formation, and this region has been suggested to form a continuous α helix

(Accola et al. 1998). This model is consistent with tomographic reconstructions of the immature lattice, which revealed rod-like structures descending toward the NC layer along the six-fold symmetry axis below CA_{CTD} (Fig. 4B; Wright et al. 2007). These features can be modeled as six helix bundles, although this model again awaits definitive testing at higher resolution. Interestingly, the helical propensity of the CA-SP1 junction sequence is rather weak (Morellet et al. 2005), but recent evidence suggests that helix formation is strongly induced by molecular crowding (Datta et al. 2011b). Thus, helix formation could provide a switch that helps trigger immature lattice formation as the Gag molecules coalesce (Datta et al. 2011b). Subsequent proteolytic cleavage at the CA-SP1 junction would then destroy the helix during maturation, destabilize immature lattice interactions, and help drive conversion to the mature capsid lattice.

In addition to defining the structure of Gag hexamers, the cryo-EM tomographic studies revealed how the hexameric lattice curves into a spherical structure. A perfect hexagonal lattice lacks declination, and must therefore include nonhexameric defects in order to enclose space. In the mature capsid, this is achieved by including 12 pentameric defects (see below). In contrast, the immature lattice contains small, irregularly shaped defects and holes that permit it to curve (Fig. 4A; Briggs et al. 2009). Nevertheless, the Gag shell forms a contiguous lattice rather than consisting of smaller "islands" of regular Gag arrays. It does contain one large gap, however, which covers approximately one-third of the surface area of the membrane (Wright et al. 2007; Briggs et al. 2009). This gap in the Gag lattice is created when the virus buds (Fig. 4A), because Gag assembly and budding appear to be competitive processes, and immature virions typically bud before the Gag molecules have finished polymerizing into fully closed shells (Carlson et al. 2010). As a result of this gap, virions contain fewer Gag molecules than was initially calculated based on the assumption of a complete virus shell. The precise number of Gag molecules depends on the size of the individual virion and the completeness of the Gag shell, with a virion of 130 nm diameter containing roughly 2500 Gag molecules (Carlson et al. 2008).

HIV BUDDING

Late Domains and ESCRT Pathway Recruitment

Although the viral Gag protein is responsible for cofactor packaging and virion assembly, the virus usurps the host ESCRT pathway to terminate Gag polymerization and catalyze release (Morita and Sundquist 2004; Bieniasz 2009; Carlton and Martin-Serrano 2009; Usami et al. 2009; Hurley and Hanson 2010; Peel et al. 2011). ESCRT factors also catalyze the topologically equivalent membrane fission reactions that release vesicles into endosomal multivesicular bodies (Hurley and Hanson 2010; Peel et al. 2011) and that separate daughter cells during the abscission stage of cytokinesis (Carlton and Martin-Serrano 2007, 2009; Hurley and Hanson 2010; Elia et al. 2011; Guizetti et al. 2011; Peel et al. 2011). This ability of the ESCRT machinery to facilitate membrane fission from within the necks of thin, cytoplasm-filled membrane vesicles and tubules explains why HIV-1, and many other enveloped viruses, have evolved to use the pathway to bud from cells.

HIV-1 $p6^{Gag}$ contains two different "late domain" motifs that bind and recruit early-acting ESCRT factors (Morita and Sundquist 2004; Bieniasz 2009; Carlton and Martin-Serrano 2009; Usami et al. 2009). The primary "PTAP" late domain binds the TSG101 subunit of the heterotetrameric ESCRT-I complex (Garrus et al. 2001; Martin-Serrano et al. 2001; VerPlank et al. 2001; Demirov et al. 2002; Morita and Sundquist 2004; Bieniasz 2009). Each of the four residues (Pro-Thr/Ser-Ala-Pro) makes specific contacts within an extended groove on the amino-terminal ubiquitin E2 variant (UEV) domain of TSG101 (Pornillos et al. 2002; Im et al. 2010). PTAP motifs are also found within HRS and related proteins that recruit ESCRT to endosomal membranes (Ren and Hurley 2011). Thus, the HIV-1 $p6^{Gag}$ PTAP late domain mimics a cellular ESCRT-I

recruiting motif, and Gag and HRS can both be viewed as membrane-specific adaptors for the ESCRT pathway (Pornillos et al. 2003).

The second $p6^{Gag}$ late domain, designated "YPXL" (Tyr-Pro-X-Leu, where "X" can vary in sequence and length), binds the ESCRT factor ALIX (Strack et al. 2003; Usami et al. 2009). The YPXL late domain contributes significantly to HIV-1 replication (Fujii et al. 2009; Eekels et al. 2011) but is less critical than the PTAP motif in most cell types. Retroviral YPXL late domains exhibit considerable sequence variation, but a conserved tyrosine binds deep within a pocket on the second arm of the ALIX V domain in all cases, and downstream hydrophobic residues contact ALIX along a shallow adjacent groove (Zhai et al. 2008, 2011). Once again, the virus is mimicking a motif used by cellular ALIX ligands in fungi (Vincent et al. 2003), although YPXL-containing binding partners for mammalian ALIX proteins remain to be characterized. The amino-terminal ALIX Bro domain also interacts with NC^{Gag}, and NC mutants can exhibit budding defects, reflecting the apparent functional importance of this interaction (Popov et al. 2008, 2009; Dussupt et al. 2009). Finally, the carboxy-terminal domain of CA^{Gag} interacts with NEDD4L, a member of the human NEDD4 ubiquitin E3 ligase protein family (Chung et al. 2008; Usami et al. 2008; Weiss et al. 2010). Although this interaction contributes only modestly to HIV-1 budding, NEDD4 family members play critical roles in the budding of other retroviruses through direct interactions with their PPXY (Pro-Pro-X-Tyr) late domains (Morita and Sundquist 2004; Bieniasz 2009; Carlton and Martin-Serrano 2009; Usami et al. 2009).

Assembly of the Core ESCRT Machinery

The human ESCRT pathway comprises more than 30 different proteins, and this complexity is expanded further by associated regulatory and ubiquitylation machinery. Essential ESCRT pathway functions and mechanisms are conserved across eukaryotes and archaea, and many basic principles have been elucidated through biochemical and genetic analyses of the simpler yeast pathway (Saksena et al. 2007; Hurley and Hanson 2010). Recent functional studies have identified a minimal core set of human ESCRT machinery that is essential for HIV-1 budding (Fig. 5). In essence, TSG101/ESCRT-I and ALIX both function by recruiting downstream ESCRT-III and VPS4 complexes, which in turn mediate membrane fission and ESCRT factor recycling (Morita and Sundquist 2004; Bieniasz 2009; Carlton and Martin-Serrano 2009; Hurley and Hanson 2010; Peel

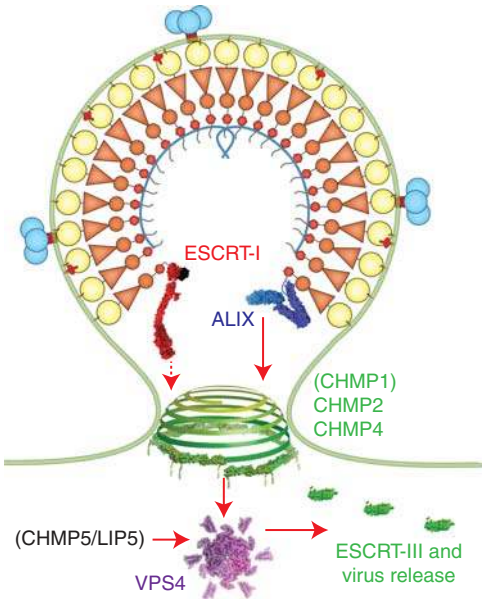

Figure 5. Summary of the essential core ESCRT machinery used in HIV-1 budding (with auxiliary factors shown in parentheses), illustrating a leading model for the budding mechanism. Late domain motifs within $p6^{Gag}$ bind directly to the UEV domain of the TSG101 subunit of the heterotetrameric ESCRT-I complex (red, with bound ubiquitin in black, 1S1Q, 2P22) and the V domain of ALIX (blue, 2OEV). These interactions result in the recruitment of the ESCRT-III proteins of the CHMP1, CHMP2, and CHMP4 families (green, 2GD5), which apparently polymerize into a "dome" that promotes closure of the membrane neck (Peel et al. 2011). They also recruit the VPS4 ATPases (purple, 1XWI, 1YXR), which completes the membrane fission reaction and uses the energy of ATPase to release the ESCRT-III from the membrane and back into the cytoplasm. See text for details.

et al. 2011). Humans have 12 different ESCRT-III-like proteins (predominantly known by "CHMP" designations), which can be subdivided into seven families. These proteins share a common architecture, with an amino-terminal core domain comprising an extended four-helix bundle (Muziol et al. 2006; Bajorek et al. 2009; Xiao et al. 2009), and carboxy-terminal tails that can fold back and autoinhibit core oligomerization (Lin et al. 2005; Zamborlini et al. 2006; Lata et al. 2008a; Bajorek et al. 2009). Only the CHMP2 and CHMP4 families play critical functional roles in HIV-1 budding, although CHMP1 and CHMP3 family members may also contribute modestly (Jouvenet et al. 2011; Morita et al. 2011). The ESCRT-I interactions that lead to CHMP2/CHMP4 recruitment are not yet clear, but the ALIX branch of the pathway is better understood. As illustrated in Figure 5, the following sequence of events is consistent with current analyses of ESCRT pathway recruitment. (1) Late domain binding induces the soluble ALIX protein to undergo a conformation change that leads to dimerization and activates ALIX for membrane binding and ESCRT-III recruitment (Pires et al. 2009; Usami et al. 2009). (2) The Bro domain of the activated ALIX protein binds carboxy-terminal helices located within the tails of all three human CHMP4 proteins (McCullough et al. 2008). (3) This interaction relieves CHMP4 autoinhibition and induces the protein to polymerize into filaments within the virion neck (Hanson et al. 2008; Pires et al. 2009; Usami et al. 2009; Hurley and Hanson 2010; Peel et al. 2011). (4) CHMP4 filaments then recruit (or copolymerize with) the CHMP2 proteins (Lata et al. 2008b; Morita et al. 2011). (5) Deposition of CHMP2 exposes the protein's carboxy-terminal tail, which contains a helical sequence motif that binds the amino-terminal MIT domains of VPS4 ATPases (Obita et al. 2007; Stuchell-Brereton et al. 2007; Hurley and Yang 2008). (6) The recruited VPS4 proteins assemble into enzymatically active higher order complexes (Babst et al. 1998; Scott et al. 2005; Shestakova et al. 2010). Like most other AAA ATPases, VPS4 forms hexameric rings, and the active enzyme appears to comprise two stacked, inequivalent hexameric rings (Yu et al. 2008) (although alternative models have been proposed). Each virus budding site recruits approximately three to five VPS4 dodecamers (Baumgartel et al. 2011), which may be linked together through bridges composed of the CHMP5/LIP5 activator complex (Yang and Hurley 2010). The entire ESCRT assembly process takes approximately 10 minutes and occurs in multiple stages, with a gradual and concomitant buildup of the Gag and ALIX proteins, followed by short (~2 min) bursts of ESCRT-III and VPS4 recruitment immediately prior to virus budding (Baumgartel et al. 2011; Jouvenet et al. 2011).

Models for Membrane Fission

The detailed mechanism of ESCRT-mediated membrane fission is an active research frontier, but several important aspects of the process have recently emerged. CHMP4 subunits, possibly in complex with CHMP2 and other ESCRT-III proteins, appear to form spiraling filaments within the neck of the budding virus (Fig. 5; Ghazi-Tabatabai et al. 2008; Hanson et al. 2008; Lata et al. 2008b; Teis et al. 2008; Saksena et al. 2009; Wollert et al. 2009; Elia et al. 2011; Guizetti et al. 2011). As the filaments spiral inward, they may create closed "domes" that constrict the opposing membranes and promote fission (Fabrikant et al. 2009; Hurley and Hanson 2010; Peel et al. 2011). VPS4 also apparently plays an active role in the membrane fission reaction because the enzyme is recruited immediately prior to virus budding (Baumgartel et al. 2011; Elia et al. 2011; Jouvenet et al. 2011), and because ESCRT-III recruitment alone is insufficient for virus release (Jouvenet et al. 2011). Possible roles for VPS4 include helping to promote ESCRT-III dome formation and/or removing ESCRT-III subunits from the dome, thereby destabilizing hemi-fission intermediates and helping to drive fission to completion (Hanson et al. 2008; Lata et al. 2008b; Baumgartel et al. 2011; Peel et al. 2011). In the final stage of the cycle, VPS4 uses the energy of ATP hydrolysis to disassemble the filaments and release the ESCRT-III subunits back into the cytoplasm as soluble, autoinhibited

proteins (Babst et al. 1998; Ghazi-Tabatabai et al. 2008; Lata et al. 2008b; Wollert et al. 2009; Davies et al. 2010). Thus, the energy required for virus budding is provided by ATP hydrolysis, in the form of VPS4-mediated protein disassembly and refolding.

Blocks to HIV-1 Release

HIV-1 budding and release are essential for spreading viral infection, and it is therefore not surprising that innate immune pathways have evolved to interfere with these processes. It is now well established that the antiviral protein tetherin blocks HIV-1 dissemination by tethering newly budded viral particles to the cell surface. Tetherin is antagonized by the HIV-1 Vpu protein, and this remarkable restriction system is described in detail in Malim and Bieniasz (2011). Recently, Leis and colleagues have reported that another interferon-inducible protein, ISG-15, can inhibit HIV-1 release at the earlier budding stage by interfering with ESCRT-III protein activities. ISG-15 is a ubiquitin-like protein that can be covalently attached to the lysine side chains of ESCRT-III subunits (Skaug and Chen 2010). ISGylation of CHMP5 and other ESCRT-III subunits appears to impair VPS4 function by sequestering the LIP5-CHMP5 activator away from the enzyme and by reducing VPS4 recruitment and activity (Pincetic et al. 2010; Kuang et al. 2011). It will now be important to determine the relative contributions of these activities in inhibiting HIV-1 replication in vivo and how the virus overcomes such blocks.

HIV MATURATION

Architecture of the Mature HIV-1 Virion

Viral maturation begins concomitant with (or immediately following) budding, and is driven by viral PR cleavage of the Gag and Gag-Pro-Pol polyproteins at ten different sites, ultimately producing the fully processed MA, CA, NC, p6, PR, RT, and IN proteins (Fig. 1A–C; see online Movie 3 at www.perspectivesinmedicine.org; Swanstrom and Wills 1997; Hill et al. 2005). Over the course of maturation, these processed proteins rearrange dramatically to create the mature infectious virion, with its characteristic conical core (Fig. 1D–G). MA remains associated with the inner leaflet of the viral membrane, forming a discontinuous matrix shell that lacks long-range order. The outer capsid shell of the core particle is composed of approximately 1200 copies of CA and is typically conical, although tubes and other aberrant assemblies, including double capsids, also form at lower frequencies (Briggs et al. 2003b; Benjamin et al. 2005). The capsid approaches the matrix closely at both ends (Benjamin et al. 2005; Briggs et al. 2006), particularly at the narrow end, which may represent the nucleation site for assembly (Briggs et al. 2006). The capsid surrounds the nucleocapsid, which typically resides at the wide end of the capsid and lacks obvious long-range order (Briggs et al. 2006).

The Viral Capsid

The capsid performs essential functions during the early stages of HIV-1 replication, although these functions are not yet fully understood in mechanistic detail. In newly infected cells, the capsid interacts with both positive-acting host factors like cyclophilin A and transportin-3, and with restriction factors of the TRIM5-α family (Luban 2007; Sebastian and Luban 2007; Yamashita and Emerman 2009; Krishnan et al. 2010). CA mutations that block capsid assembly or destabilize the capsid typically inhibit reverse transcription, implying that the capsid helps to organize the replicating genome (Forshey et al. 2002). Conversely, mutations that hyperstabilize the capsid also inhibit reverse transcription, implying that the capsid must disassemble or uncoat in a timely fashion (Forshey et al. 2002). CA mutations can also inhibit or alter nuclear localization, indicating that the capsid (or at least CA subunits) probably play important roles in nuclear targeting and/or import of the preintegration complex (Dismuke and Aiken 2006; Yamashita et al. 2007; Lee et al. 2010).

HIV-1 capsids are geometric structures called "fullerene cones" (Fig. 6A), which are a family of related structures comprising conical

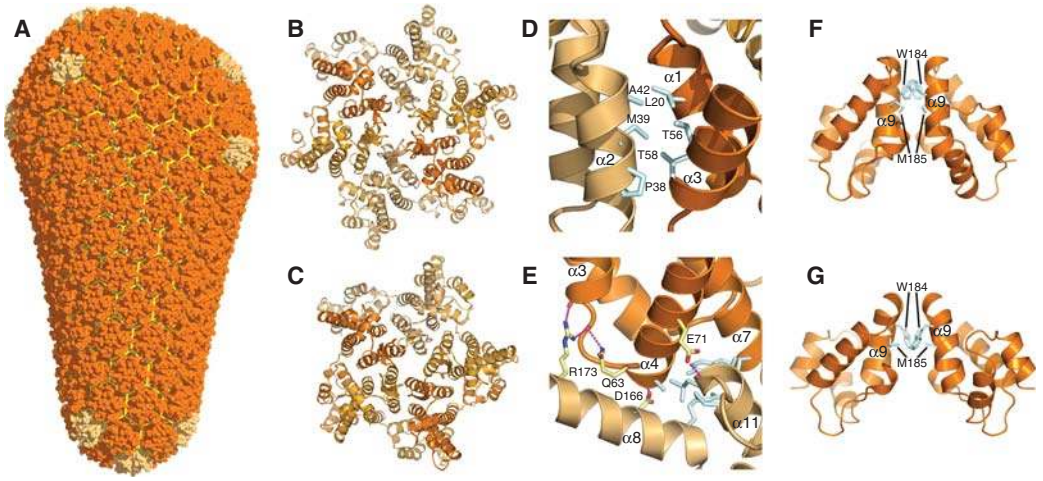

Figure 6. Fullerene cone model for the HIV-1 capsid. (*A*) Molecular model of the HIV-1 capsid, with CA hexamers in orange and pentamers in tan (adapted from Pornillos et al. 2011). (*B*) Structure of the HIV-1 CA hexamer (3H47). (*C*) Structure of the HIV-1 CA pentamer (3P05). (*D*) Detailed structure of the CA_{NTD}–CA_{NTD} interface that stabilizes the hexameric ring. Hydrophobic residues that stabilize the interaction between CA helices 1, 2, and 3 are highlighted. (*E*) Detailed structure of the CA_{NTD}(orange)–CA_{CTD}(tan) interface that forms a "girdle" around the hexameric and pentameric rings. Interface residues are highlighted, as are a series of salt bridges and hydrogen bonds that stabilize the interface while allowing it to "swivel" in response to changes in lattice curvature. (*F,G*) Two alternative structures of the CA_{CTD} dimer, 1A43and 2KOD. Two key interface residues (W184 and M185) are shown in each case to emphasize the fact that similar CA surfaces are used in both dimers.

hexagonal nets that close at both ends through the introduction of 12 pentagonal defects (Ganser et al. 1999; Ganser-Pornillos et al. 2008). Thus, unlike icosahedral viruses, HIV-1 capsids are best viewed as a continuum of related structures rather than as a single unique assembly. Most authentic HIV-1 capsids exhibit the 19.2° cone angle required by conical hexagonal packing, but their overall lengths and cap shapes vary owing to the insertion of pentagons at different positions in the hexagonal net (Benjamin et al. 2005; Briggs et al. 2006). Positioning of pentamers at alternate sites also accounts for the different capsid shapes seen in other retroviruses because "tubular" capsids are created when the 12 pentamers are symmetrically distributed at either end of the tube, and "spherical" capsids are created when the pentamers are distributed more evenly throughout the hexagonal net (Ganser-Pornillos et al. 2004).

A series of different intersubunit CA interactions stabilize the capsid lattice. The CA subunits are organized into hexameric and pentameric rings, and both of these structures have now been visualized crystallographically (Fig. 6B,C; see online Movie 4 at www.perspectivesinmedicine.org; Pornillos et al. 2009, 2011). The organization of subunits within the two types of rings is remarkably similar, and HIV-1 CA hexamers and pentamers are therefore an excellent example of the principle of quasi-equivalence as originally envisioned by Caspar and Klug (1962). In both cases, the rings are stabilized by interactions between the first three helices of CA_{NTD} (Fig. 6D). The CA rings are buttressed by an exterior "girdle" formed by CA_{CTD}, with each CA_{CTD} domain contacting the CA_{NTD} of a neighboring subunit in the ring (Fig. 6B,C,E). CA_{CTD} also makes important inter-ring contacts across the local two- and three-fold axes, thereby stabilizing the extended lattice (Ganser-Pornillos et al. 2007; Byeon et al. 2009). Isolated CA_{CTD} polypeptides can make two different types of two-fold symmetric dimers which utilize the same basic interface but differ in their detailed

packing interactions, and both of these dimers may participate in capsid assembly (Fig. 6F,G; Gamble et al. 1997; Ganser-Pornillos et al. 2007; Byeon et al. 2009).

Fullerene cones lack symmetry, which implies that all of the different CA subunits must reside in distinct local environments. The individual CA subunits must therefore have sufficient flexibility to accommodate at least three different types of heterogeneity. Both CA hexamers and pentamers can be formed because the interface between subunits in the hexameric ring is highly hydrated, and mobile water molecules are well suited for adjusting to the subtle changes required to make the pentamer interface. Alterations in lattice curvature across different regions of the cone surface are accommodated by flexibility in the NTD-CTD linker and in the NTD–CTD interface (Fig. 6E), which allows the two CA domains to move relative to one another. Finally, differences in inter-ring packing interactions may be accommodated by using both types of CTD-CTD dimers at different positions of the cone (Pornillos et al. 2011).

The Viral Protease

HIV-1 PR is one of the most extensively characterized proteins in molecular biology owing to its importance as a drug target (Wlodawer and Gustchina 2000; Louis et al. 2007; Ali et al. 2010; and see Arts and Hazuda 2011). Like other aspartic acid proteases, HIV-1 PR uses two aspartic acid side chains within a characteristic Asp-Thr-Gly "fireman's grip" motif to activate the nucleophilic water molecule that cleaves the peptide bond (Cooper 2002). Retroviral proteases are unusual, however, in that the active enzyme is a dimer of two identical subunits. The active site transverses the dimer interface, stabilized by the fireman's grip and by a four-stranded mixed β-sheet created by the amino and carboxyl termini of each subunit. Two extended flexible loops lie on the other side of the active site, and these "flaps" open to allow substrates access to the active site (Fig. 1H).

The mechanism by which PR is activated during Gag assembly and budding is still not fully understood. PR is essentially inactive within the Gag-Pro-Pol polyprotein because the mature active enzyme is a dimer, whereas unprocessed PR constructs with amino-terminal extensions dimerize only very weakly. However, such constructs can form transient dimeric "encounter" complexes. A small fraction of these encounter complexes have enzymatic activity and can accommodate insertion of the amino-terminal cleavage site into the substrate-binding cleft, which can lead to autoprocessing and formation of a stable dimer with full catalytic activity (Tang et al. 2008). Gag trafficking probably helps to regulate PR activation by preventing premature PR dimerization until the Gag molecules coalesce at the plasma membrane. Consistent with this idea, artificially dimerized PR subunits are activated prematurely, which inhibits particle production (Krausslich et al. 1991). Assembly-mediated PR dimerization is unlikely to account for the entire activation mechanism, however, because Mason-Pfizer monkey virus and other betaretroviruses preassemble into immature particles in the cytoplasm, yet their PR enzymes are not activated until they are transported and bud from the plasma membrane (Parker and Hunter 2001). Thus, PR must be activated by additional mechanisms, at least in the betaretroviruses.

During viral maturation, PR cleaves five different sites within Gag (Fig. 1B) and five different sites within Gag-Pro-Pol (not shown). Schiffer and colleagues have determined crystal structures of HIV-1 PR in complex with peptides that correspond to seven of these cleavage sites (Prabu-Jeyabalan et al. 2002, 2004). Binding of asymmetric substrates breaks the two-fold symmetry, so that the two PR subunits are no longer equivalent. Substrates bind in extended, β-strand conformations and make sheet-like interactions with the flaps and the base of the PR active site. The eight side chains from four residues on either side of the scissile bond bind in a series of six different enzyme pockets (with the first and third residues on either side of the scissile bond sharing common pockets). The enzyme appears to recognize the overall shape of the substrate rather than its specific sequence, as evidenced by the fact that the

different Gag and Gag-Pro-Pol cleavage sites vary considerably in amino acid sequence, yet all bind within the enzyme with very similar steric footprints (Prabu-Jeyabalan et al. 2002).

Maturation Dynamics

Important changes that occur during HIV-1 maturation include activation of the fusogenic activity of the viral Env protein (Murakami et al. 2004; Wyma et al. 2004), capsid assembly, stabilization of the genomic RNA dimer, and rearrangement of the $tRNA^{Lys,3}$ primer–genome complex (Moore et al. 2009; Rein 2010). Maturation is a dynamic, multistep process that involves a series of conformational switches and subunit rearrangements. Temporal control of viral maturation is provided, at least in part, by the very different rates of processing at the five Gag processing sites, whose cleavage rates vary by up to 400-fold. These cleavage sites fall into three different categories: rapid (SP1/NC), intermediate (SP2/p6, MA/CA), and slow (NC/SP2, CA/SP1) (Pettit et al. 1994). Analyses of mutant virions with blockages at the different Gag sites indicate that each cleavage event performs a different function. SP1/NC cleavage activates Env (Wyma et al. 2004) and promotes condensation of the RNP particle (de Marco et al. 2010), SP2 processing frees NC to chaperone formation of the stable genomic RNA dimer (Kafaie et al. 2008; Ohishi et al. 2011), MA/CA cleavage disassembles the immature lattice and releases CA-SP1, and CA-SP1 cleavage frees CA to form the conical capsid (de Marco et al. 2010). In at least some cases, Gag proteolysis induces local conformational changes that favor alternative protein–protein interactions. For example, cleavage at the MA/CA site causes the first 13 CA residues, which are disordered in the MA-CA protein, to fold into a β-hairpin that packs above the first three CA_{NTD} helices and promotes the formation of mature CA hexamers (Gitti et al. 1996; Tang et al. 2002). Cleavage at the CA-SP1 junction also appears to trigger a conformational rearrangement (Gross et al. 2000), although the molecular details of this transition are not yet well understood.

SITES OF VIRUS RELEASE AND CELL-TO-CELL TRANSMISSION

HIV-1 Assembles and Buds at the Plasma Membrane

The general view that HIV-1 assembles and buds at the plasma membrane of the infected cell was challenged by electron microscopy studies reporting budding sites and released virions in apparently intracellular compartments in macrophages and other cell lines. These compartments were reactive for late endosomal markers, which—in combination with the ESCRT requirement for virion release—led to the suggestion that HIV buds into multivesicular bodies and is subsequently released from the cell via vesicular transport and fusion at the plasma membrane (Pelchen-Matthews et al. 2003). This concept is incompatible with several more recent results, however, showing that newly synthesized Gag reaches the plasma membrane before it accumulates at late endosomes and that endosomal accumulation can be inhibited without effect on virus release (Jouvenet et al. 2006). More importantly, fluorescence microscopy analysis of HIV-1 formation in living cells (Jouvenet et al. 2008; Ivanchenko et al. 2009) only detected single particle assembly at the plasma membrane, inconsistent with vesicular release of preassembled virions from a vesicular compartment. The apparently intracellular sites of HIV-1 assembly and budding in macrophages were ultimately shown to be deep plasma membrane invaginations that were connected to the surface via narrow channels, but were nevertheless accessible to membrane-impermeant stains (Deneka et al. 2007; Welsch et al. 2007) and traceable through ion-abrasion scanning electron microscopy (Bennett et al. 2009). Thus, HIV-1 assembly and budding appears to occur predominantly at the plasma membrane in all physiologically relevant cells.

The Virological Synapse

Polarized HIV-1 release was initially observed in electron microscopy studies that reported accumulation of HIV-1 budding at sites of

cell-to-cell contact (Phillips and Bourinbaiar 1992). These observations were consistent with the idea that direct cell-to-cell spread of HIV-1 via specialized cell contacts is much more efficient than infection with cell-free virus and is thus likely to represent the predominant mode of virus spread in vivo, particularly within lymphoid tissues. Microscopic studies of $CD4^+$ T-cell cultures later revealed close connections between infected donor cells and uninfected target cells, and cell-to-cell transmission of virions across these contact zones, which were termed virological synapses (VS) (Piguet and Sattentau 2004; Mothes et al. 2010; Jolly and Sattentau 2004; Haller and Fackler 2008). This name was chosen because the structures were reminiscent of immunological synapses, even though the two structures are clearly distinct in terms of protein composition, signaling, and dynamics (Vasiliver-Shamis et al. 2010). Multiple contact zones between one HIV-infected $CD4^+$ T cell and several uninfected cells were also observed and have been termed polysynapses (Rudnicka 2009). VS have been described between infected and uninfected $CD4^+$ T cells, between macrophages and $CD4^+$ T cells, and between virus-exposed dendritic cells and $CD4^+$ T cells. Although all of these contact zones are likely to serve analogous functions in enhancing virus transmission, there are some fundamental differences: In VS between T cells, polarized HIV-1 release and subsequent target cell entry occurs across the VS (Piguet and Sattentau 2004), whereas in macrophages virions that have accumulated within the invaginated compartment (see above) are brought toward the contact zone (Gousset et al. 2008). Mature DCs capture cell-free virions into a compartment that stains for late endosomal markers, with subsequent transport and release toward the cell contact zone. The ultrastructure of this contact zone has been analyzed by ion abrasion scanning electron microscopy and electron tomography, which show a close envelopment of the T cells into DC-derived sheet-like membrane extensions, sometimes containing T-cell filopodia. This close interaction provides a shielded environment that allows CD4-dependent transfer of sequestered virions from DCs to the T-cell surface and subsequent infection (Felts et al. 2010).

Viral and cellular components that polarize on the producer cell of the T-cell VS include HIV-1 Gag and Env, cellular tetraspanins, and the microtubule organizing center (MTOC), whereas HIV-1 receptors (CD4 and coreceptors) and the actin cytoskeleton polarize on the target cell (Haller and Fackler 2008). VS formation is dependent on HIV-1 Env proteins on the producer cell and viral receptors on the target cell, and is therefore a virus-induced structure. The contact zone in the producer cell is enriched in "raft" marker proteins and the VS is destroyed by cholesterol depletion, suggesting that membrane microdomains also make important contributions. Polarization and VS formation are dependent on an intact microtubule and actin cytoskeleton, and the VS can be stabilized by interactions of the cellular adhesion molecules ICAM-1 and LFA-1 (Jolly et al. 2007). ZAP-70, a kinase that regulates T-cell signaling and immunological synapse formation, is also required for polarized localization of HIV structural proteins, VS formation, and efficient cell-to-cell spread (Sol-Foulon et al. 2007).

The intracellular tail of the TM protein appears to be essential for polarized targeting of Env and Gag as well as for efficient spread of both murine leukemia virus (Jin et al. 2009) and HIV-1 (Emerson et al. 2010). HIV-1 Gag localizes to the trailing end of polarized T cells, termed the uropod, and this localization appears to depend on the formation of higher order oligomers and requires the NC domain (Llewellyn et al. 2010).

Despite their different names, infections that occur via cell-to-cell transfer and via cell-free particles both involve production of cell-free virions and likely involve fundamentally similar molecular mechanisms of virus assembly, budding, and release. Electron microscopy and tomographic (Martin et al. 2010) studies of VS revealed HIV budding events and numerous virions within the T-cell VS, which appeared to be loosely structured and to have few points of immediate contact, suggesting that the structure is relatively permeable. Transfer of viral material through the VS was

observed by video microscopy (Hubner et al. 2009) and reportedly involved micrometer-sized Gag-positive structures, which are far larger than individual virions, whereas parallel EM images showed individual HIV-1 budding sites within in the contact zone. It is unclear, therefore, whether the observed transfer events corresponded to HIV-1 infections or to transfer of vesicular material destined for lysosomal destruction. A cautious interpretation of these observations is also warranted because only a low number of actual infections were observed despite the presence of numerous budding sites and virions in the contact zone.

In principle, the VS could enhance HIV-1 transmission in several different ways. Virion release within a relatively closed environment and in the immediate vicinity of a target cell surface enriched in entry receptors provides one obvious advantage over cell-free infections, which are limited by extracellular diffusion and random cell surface attachment. Cell-to-cell contacts between uninfected and infected cells may also induce signaling pathways and activate the target cell, thus making it more permissive for HIV-1 infection. Finally, several studies have suggested that the VS could shield newly produced virions from circulating neutralizing antibodies and entry inhibitors (Chen et al. 2007; Hubner et al. 2009). However, those reports have not been confirmed in more recent studies (Massanella et al. 2009; Martin et al. 2010) and also seem inconsistent with ultrastructural analyses that suggest a relatively loose contact zone. Thus, the VS appears not to confer protection from neutralization or chemotherapeutic agents.

Membrane Bridges and Cell-to-Cell Transmission

Besides VS, several types of membrane bridges have been reported to enhance cell-to-cell transmission of HIV-1 and other retroviruses (Sherer and Mothes 2008; Mothes et al. 2010). Such structures include filopodial bridges, termed cytonemes, which have been shown to support the spread of murine leukemia virus (Sherer 2007), and membrane nanotubes, which can link $CD4^+$ T cells and support HIV transfer (Sherer and Mothes 2008; Sowinski et al. 2008). Importantly, virion transport again occurs on the outer surfaces of these membrane connections, and therefore apparently involves normal budding and receptor-mediated entry processes. Although these structures are certainly intriguing, their relative contributions to retrovirus spread in vivo are difficult to quantify.

PERSPECTIVES

Despite considerable progress, a number of key aspects of HIV-1 assembly, budding, and maturation are not yet well understood and represent important research frontiers. The most pressing issues that need to be addressed include (1) determining the structure of the immature Gag lattice at high resolution, (2) defining the precise mechanism of membrane fission during virus budding, (3) elucidating the mechanism of PR activation, (4) defining the different stages of viral maturation in molecular detail, and (5) characterizing viral capsid functions and the mechanism of capsid uncoating during the early stages of the viral life cycle. Significant progress has already been made in identifying small molecule inhibitors of capsid assembly, budding, and maturation (Adamson et al. 2009; Blair et al. 2009; Jiang et al. 2011), and these efforts will undoubtedly be aided by a greater understanding of the molecular virology, biochemistry, and structural biology of these important viral processes.

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