Published in final edited form as:
Physiology (Bethesda). 2011 August ; 26(4): 236–251. doi:10.1152/physiol.00051.2010.

New Insights into HIV Assembly and Trafficking

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Abstract

Assembly and release of human immunodeficiency virus type 1 (HIV-1) particles is mediated by the viral Gag polyprotein precursor. Gag is synthesized in the cytosol and rapidly translocates to membrane to orchestrate particle production. The cell biology of HIV-1 Gag trafficking is currently one of the least understood aspects of HIV-1 replication. In this review, we highlight the current understanding of the cellular machinery involved in Gag trafficking and virus assembly.

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent of acquired immune deficiency syndrome (AIDS). HIV-1 specifically targets the human immune system, primarily infecting CD4+ helper T cells, macrophages, and dendritic cells. HIV-1 infection destroys CD4+ cells by various mechanisms, leading to a general weakening of the immune system, the onset of opportunistic infections, and, ultimately, death. Since its discovery nearly three decades ago, HIV-1 infection has claimed more than 25 million lives globally (156). Currently, 33 million people are infected with HIV-1, with sub-Saharan Africa bearing much of the brunt (227). No vaccine to prevent HIV-1 infection is available (129) and antiretroviral drugs that disrupt different stages of the virus life cycle remain the only viable treatment option (22). However, HIV's replication strategy ensures rapid generation of drug-resistant mutant viral variants (4, 150, 176). To limit the emergence of drug-resistant strains, a highly active antiretroviral therapy (HAART) that involves simultaneous treatment with a cocktail of antiretrovirals is the standard of care (26, 87, 92, 213). Prolonged HAART treatment leads to adverse effects in a significant proportion of patients, which in turn contributes to patient nonadherence (95, 153, 201, 239). Furthermore, HAART does not eradicate HIV-1 infection because a stable viral reservoir is archived in the resting memory CD4+ T cells (6, 37, 39, 76, 130, 200, 225). Thus there is an urgent necessity to develop novel antiretrovirals that target additional crucial steps in the HIV-1 replication cycle (1, 3). The late stages of the HIV-1 replication cycle, specifically virus assembly and release, have not been successfully exploited for antiviral therapy (5). The major viral structural protein Gag coordinates particle assembly and release, and successful particle production critically depends on highly regulated intracellular trafficking of Gag to appropriate assembly sites (2, 119). In this review, we highlight recent findings that have generated new insights into HIV-1 trafficking, assembly, and release.

HIV-1 Replication: An Overview

HIV-1 replication in infected cells is intricately connected to host cell biology and progresses through a series of finely choreographed steps, broadly categorized into early and late events (27, 64, 69, 81). Early events begin when HIV-1 binds to the host cell CD4

No conflicts of interest, financial or otherwise, are declared by the author(s).

receptor and then one of the chemokine coreceptors, CCR5 or CXCR4. Subsequently, viral and cellular membranes fuse, the viral "core" enters the cytoplasm, the viral enzyme reverse transcriptase (RT) copies the viral RNA (vRNA) genome into a DNA copy that then traffics into the nucleus, where the viral integrase (IN) catalyzes the integration of that DNA copy into the host chromosome to generate the integrated provirus (97, 171, 191, 218, 230). During the subsequent late events, the host RNA polymerase II transcribes the proviral DNA back into viral mRNAs. Full-length (unspliced), singly spliced, and multiply spliced vRNAs are then transported to the cytoplasm and translated into several structural polyprotein precursors [the Gag precursor Pr55Gag, the Gag-Pol precursor Pr160GagPol, and the envelope (Env) glycoprotein precursor gp160] and several accessory and regulatory proteins (69). A subset of these viral proteins, including Gag, GagPol, and Env, traffic to membranes where they assemble into a new generation of virus particles (73, 119, 199). The resultant non-infectious, immature virus-like particles (VLPs) bud and finally pinch off from the membrane (15, 43, 157, 228); during particle release, they undergo a process termed maturation, whereby the viral protease (PR) cleaves the Gag and GagPol precursor polyproteins into mature proteins, leading to the formation of the mature conical core. Virus maturation is essential for particle infectivity (5).

HIV-1 Gag: An Assembly Machine

HIV-1 employs an efficient strategy to synthesize progeny particles whereby the Pr55Gag precursor (FIGURE 1), often referred to simply as Gag, is used as a building block and then is cleaved by PR only after assembly has been completed (2, 65). Pr55Gag is necessary and sufficient for orchestrating particle assembly, since its expression alone in suitable eukaryotic cells leads to the production of VLPs (77). HIV-1 Gag is cotranslationally myristylated and comprises four major domains [matrix (MA), capsid (CA), nucleocapsid (NC), and p6] and two spacer peptides (SP1 and SP2) (65) (FIGURE 1). The NH_2-terminal myristate moiety promotes membrane binding, and a highly basic region (HBR) in MA binds the phospholipid phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2] in the inner leaflet of plasma membrane (PM), thereby stabilizing Gag-membrane association. Subsequently, CA, SP1, and NC domains cooperate to promote Gag multimerization and assembly (16). Interaction of the NC domain of Gag with the vRNA, which likely occurs initially in the cytosol (134), promotes Gag multimerization. The assembling particle incorporates the Pr160GagPol polyprotein that encodes the three viral enzymes: RT, IN, and PR. The resulting spherical protein shell induces PM curvature and forms a late-budding structure. The host cell-derived membrane that envelopes the budding virus particles bears the viral Env glycoproteins in the form of a heterotrimeric complex of the surface Env glycoprotein gp120 and the transmembrane Env glycoprotein gp41 (70). Gp120 and gp41 are derived from the Env glycoprotein precursor, gp160, which is cleaved by a furin-like cellular protease during trafficking through the Golgi apparatus. Short-peptide motifs in Gag-p6, termed "late domains" because of their role in virus release, recruit the cellular ESCRT (endosomal sorting complex required for transport) machinery, which normally assists budding and release of vesicles into the lumen of late endosomes (103), to mediate virus particle release. The immature Gag shell, in which Gag molecules are radially arranged with their NH_2 termini in contact with the membrane and their COOH termini oriented toward the center of the virion (FIGURE 2), disassembles concomitant with particle release when the viral PR cleaves the Gag and Gag-Pol polyproteins into their constituent proteins. A striking morphological change ensues to yield the mature infectious virion, wherein MA remains membrane-associated and CA molecules reassemble to form the conical core (73). The core encloses the ribonucleoprotein complex, comprising a dimer of viral genomic RNA in a complex with the NC protein and the viral enzymes RT and IN. When the released virions infect a target cell, the mature Gag proteins, along with other viral and cellular cofactors, direct the early events in the replication cycle. Roles of MA, CA, and NC in these

early events differ dramatically from the functions they execute as Gag domains, highlighting the remarkable versatility of HIV-1 Gag (65).

HIV-1 Assembly Site

The destination of nascent Gag is the particle assembly site(s), and the N-myristylated Gag assembles primarily on membranes in vivo (177, 178). Hence, plausible intracellular destinations include the inner leaflet of the PM and membrane-limited subcellular structures (FIGURE 3). Assembly at the cell periphery, i.e., the PM, ensures rapid and efficient virion dissemination, either to the extracellular milieu as cell-free virus or to the adjacent host cell via virological synapses. However, assembly at the PM also necessitates that viral components efficiently traverse the highly compartmentalized cytoplasmic space and accurately target to the PM. Indeed, in cells that are physiologically relevant targets for HIV-1 infection, such as macrophages and T lymphocytes, and also in non-target fibroblasts and epithelial cells, HIV-1 Gag and, in some instances, virions have been observed in intracellular compartments that are enriched in late endosomal markers (88, 172, 180, 184, 187, 189, 197, 214). This led to the proposition that nascent Gag is targeted to endosomal membranes for assembly, and the resultant particles bud into the lumen of late endosomes/multivesicular bodies (LE/MVB) from which they exit the cell by usurping cellular processes such as the exosome release pathway (133, 169, 172, 214). Incidentally, it is well established that retroviral budding requires the ESCRT proteins, the physiological functions of which include facilitating budding of vesicles into the endosomal lumen (15, 43, 103, 157, 228). So, where does productive HIV-1 particle assembly occur in infected cells, and is the assembly site cell type-dependent?

Gag has been observed to bind membrane within 5–10 min postsynthesis (61, 89, 181, 224). Hence, identification of virus assembly sites must account for the events that occur within this narrow window of time or else will likely miss key steps in the Gag trafficking pathway. However, many of the studies that attempted to address this issue by employing fluorescence or electron microscopy or biochemical assays used nonphysiological tissue culture cell lines (HeLa, COS, and HEK293T), non-authentic Gag derivatives that were Rev-independent, codon-optimized for higher expression in mammalian cells, and tagged with fluorescent proteins exhibiting slow maturation kinetics. Not surprisingly, the use of different model cell types and Gag expression systems led to a variety of seemingly contradictory observations. For example, in living HeLa cells, nascent Gag that was stained with fluorescent biarsenic-based dyes has been visualized first at the PM (202) or instead localizing at intracellular sites before arriving at the PM (189). Similar internal Gag localization was observed using green fluorescent protein (GFP)-tagged Gag in COS cells (146, 189). In contrast, in 293T cells, GFP-tagged Gag localized first at the PM and subsequently appeared to relocate to intracellular compartments (101, 124), whereas pulse-chase experiments detected nascent Gag in both PM and LE/MVB compartments (61, 124). However, perturbing LE/MVB trafficking affected neither Gag association with the PM nor virus release. Hence, it appears that, at least in 293T cells, PM-localized virus assembly contributes predominantly to productive particle production. Recently, a similar case for HIV-1 Gag behavior in HeLa cells was made by findings from two studies that, reflecting the growing application of high-resolution live-cell microscopy to monitor single particles during highly dynamic cellular processes, used total internal reflection fluorescence microscopy (TIR-FM) to monitor the dynamics of individual HIV-1 assembly and budding events at the PM (107, 123). Although this technique permits observation of PM-associated but not intracellular events, the Gag population that contributed to productive particle production appeared to be recruited directly from the cytosol (107) and was of non-endosomal origin, whereas those Gag assemblies that were not destined to be released as

cell-free virus were associated with LE markers and appeared to have been internalized via endocytosis (123).

Efforts to clarify the HIV-1 assembly site in physiologically relevant cell types like macrophages and T cells have so far produced equally contrasting observations. Early reports indicated that a significant proportion of HIV-1 assembly in macrophages occurred in intracellular compartments that bear LE/MVB markers such as the tetraspanins (169, 180, 184, 187, 197), and indeed the released virions were enriched in several LE/MVB proteins (35). In macrophages, HIV-1 reportedly evades the progressively acidified endosomal pathway and instead accumulates in neutral-pH compartments (122). Real-time visualization of HIV-1 Gag trafficking in infected macrophages revealed that Gag accumulated at the PM and in intracellular compartments positive for LE/MVB markers (85). Interestingly, these intracellular compartments are apparently connected to the PM via microchannels that are ~20 nm in diameter and hence are actually deep invaginations of the macrophage PM (45, 238) (see FIGURE 3). These virus-sequestering compartments could potentially benefit HIV-1 in deflecting host immune responses, and being contiguous with the PM is also well suited for mediating efficient delivery of virions to uninfected T cells via cell-cell contact sites (virological synapses) (85). A study that used ion-abrasion scanning electron microscopy (IA-SEM) revealed the presence of virion-containing tubules 150–200 nm in diameter that connected the virion compartments in infected macrophages to the PM (13). Although HIV-1 assembly and release in T cells is now generally accepted as being PM-associated, recent work established that LE/MVB compartments in both T cells and macrophages can under certain circumstances support productive HIV-1 assembly; specifically, an MVB-targeted Gag mutant was released efficiently in these primary cell types (118). It is important to note that most studies have examined the localization of virus assembly in monoculture systems, which differ substantially from the in vivo situation in which many cell types are intermixed and engage in intimate contacts. As has been shown in studies of cell-cell synapses (see below), interactions between cells can have profound effects on Gag trafficking.

HIV-1 Gag Trafficking and Localization to Assembly Sites

Do HIV-1 Gag and other viral components target and assemble at specialized membrane regions? Lipid rafts, also referred to as “membrane rafts” and whose existence in vivo has been a matter of controversy (160), are highly dynamic membrane microdomains that are rich in cholesterol and sphingolipids, display asymmetric protein distribution, and compartmentalize cellular processes (58, 108, 140, 190, 217). Biochemical and microscopic data demonstrating Gag association with lipid rafts (93, 98, 138, 170, 181), and the observations that cholesterol depletion diminishes PM binding of Gag, disrupts higher-order Gag multimerization and impairs virus production (181, 183) support the hypothesis that HIV-1 assembly takes place in lipid rafts (232, 233). HIV-1 may also usurp cellular cholesterol trafficking pathways to transport Gag to virus assembly sites (221). Moreover, apparently reflecting its raft origin, the lipid composition of the HIV-1 membrane strikingly resembles that of lipid rafts and differs from host cell PM (8, 31) or bulk cellular membrane (24). The cellular phosphoinositide PI(4,5)P2, which serves as a critical determinant for Gag targeting to the PM (179) (see below; FIGURE 4), and may specifically localize to lipid rafts (99), is also concentrated in the virion membrane (31). The viral Env glycoproteins are also raft associated, apparently at least in part due to their association with Gag (14), and the gp41 cytoplasmic tail was reported to harbor the determinants for Env association with rafts (244). Incidentally, a role for the gp41 cytoplasmic tail in polarized budding of HIV-1 in nonhuman polarized epithelial cells has been proposed (142, 143, 186), suggesting that under some circumstances Env can influence Gag localization. Raft-association of the viral accessory proteins Nef and Vpu has also been reported (203, 236, 247).

How do nascent Gag and other viral components traffic to particle assembly sites? Gag synthesized in the cytosol, in principle, could traffic as monomers or lower-order oligomers to membranes, where Gag undergoes higher-order multimerization to initiate particle assembly. In vitro, soluble Gag exists in monomer-dimer or monomer-trimer equilibrium (40, 41). In vivo, Gag oligomers have been visualized by immuno-electron microscopy in the cytoplasm of insect cells (168), and nonmyristylated Gag mutants that lack membrane-binding ability form low-order multimeric structures in the cytosol (136). The identification of PI(4,5)P2 as a cellular cofactor that enables MA-mediated Gag localization to the PM (179), and subsequent characterization of this functional interaction (66, 207), provided important insights into the mechanism of Gag trafficking. Depleting cellular PI(4,5)P2 levels in the PM reduces virus release by mistargeting Gag to MVBs (179), a phenotype that resembles the behavior of Gag harboring mutations in the MA HBR (68, 180). In vitro, the NH_2-terminal myristate moiety of soluble monomeric Gag is sequestered in a hydrophobic pocket (220). However, two triggering events, Gag multimerization and interaction between the MA HBR and membrane-associated PI(4,5)P2, are believed to induce a conformational change in MA that exposes the myristate moiety (207, 220) (FIGURE 4). The MA residues Lys-29 and Lys-31 were shown to be critical for the direct interaction between MA and PI(4,5)P2 (216); these MA residues also play important roles in intracellular targeting of Gag, as mutating these amino acids redirects Gag assembly to MVBs (85, 118, 180, 182). The exposed myristate moiety subsequently inserts into the lipid bilayer, thereby stabilizing the interaction between Gag and membrane. Because both Gag multimerization and the MA HBR-PI(4,5)P2 interaction are membrane-dependent, this model necessitates the arrival of monomeric Gag molecules at the membrane. A myristylated and monomeric version of Gag was recently shown to localize, albeit at reduced levels, at the PM (51), which suggests that multimeric intermediates are not absolute prerequisites for Gag translocation to assembly sites. Indeed, a recent study combined cross-linking and membrane flotation assays to show that Gag can form low-order multimers (primarily dimers) in the cytosol but assembles into higher-order structures only at the membrane (134). The regulatory role of MA in determining the site of virus assembly is underscored by observations made with Gag mutants from which most of MA has been deleted. Although these mutant Gag proteins are competent to bind membrane and assemble virus particles, they exhibit promiscuous membrane targeting resulting in substantial amounts of virus assembly at internal membranes (56, 198, 235).

In addition to the virus-encoded elements, cellular proteins have been implicated in membrane-associated HIV-1 Gag multimerization. ABCE1 (formerly HP68), a member of the ATP-binding cassette (ABC) family of cellular ATPases, reportedly binds nascent HIV-1 Gag via the NC domain, promotes Gag oligomerization at the PM, and appears to dissociate from assembling Gag before Gag processing (49, 50, 139, 248). However, the mechanistic basis of ABCE1's ability to promote Gag assembly via formation of putative assembly intermediate complexes is unclear. Staufen1, involved in cellular RNA localization and decay, binds Gag via NC and vRNA and is incorporated into released virions in a manner that correlates with the vRNA encapsidation levels (32–34, 159). Recent findings suggest that Gag binding causes cytoplasmic staufen to relocalize to lipid rafts at the PM where it reportedly regulates Gag multimerization (152). The itinerary taken by the reported Gag-vRNA-Staufen1 complex to membrane rafts remains to be elucidated.

Several studies have implicated clathrin-associated heterotetrameric adaptor protein (AP) complexes, which assist in the sorting and transport of intracellular cargo (162, 173, 174), in Gag trafficking to assembly sites. The prominent AP complex members mediate distinct transport pathways such as between the trans-Golgi network (TGN) and endosomes (AP-1), from PM to early endosomes (AP-2), from TGN and/or endosomes to LE/MVBs (AP-3), and from TGN to lysosomes (AP-4) (173). The AP-3δ subunit was shown to interact with

HIV-1 MA and positively regulate particle production (48), whereas disrupting AP-3δ function diminished HIV-1 release in HeLa cells, apparently by obstructing Gag trafficking to LE/MVBs. The surprising finding that HIV-1 release is affected by disrupting Gag trafficking to LE/MVB in cells where HIV-1 assembly is generally considered to be PM-associated awaits further characterization. AP-3δ downregulation was also reported to disrupt particle production in dendritic cells (74). In contrast to AP-3δ, AP-2 was shown to interact with the MA-CA junction region of Gag and negatively regulate particle production (12). AP-2 also binds to Env and induces its rapid internalization from the PM (17, 28), whereas Gag has been demonstrated to suppress Env internalization from the PM presumably by masking the AP-2 binding sites in Env (53). More recently, the AP-1μ subunit was shown to directly interact with the MA domain of HIV-1 Gag and Tsg101, and like AP-3δ positively regulate virus release (29). It remains to be seen, based on a report that AP-1 traffics to TGN/endosomal membranes via direct interaction with the KIF5-binding cellular protein Gadkin (211), whether AP-1 is positioned to mediate Gag trafficking along the microtubule-based cytoskeleton. Recent work established that the GGA (Golgi-localized, γ-ear containing, Arf-binding) and Arf (ADP-ribosylation factor) proteins modulate HIV-1 assembly and release (120). GGA proteins are Arf-dependent monomeric clathrin adaptors that promote protein sorting into vesicles destined for transport to endososmes (18, 19, 78, 163), whereas Arf GTPases cycle between inactive GDP-bound and active GTP-bound forms and regulate intracellular vesicular traffic, phospholipid metabolism, and actin remodeling (38, 79, 126, 127). Humans encode three GGA proteins and five Arf proteins. Depleting GGA2 or GGA3 enhances virus release in a late domain-dependent fashion, whereas overexpressing any of the GGA proteins disrupts Gag association with membrane and inhibits particle production. Interestingly, membranous intracellular structures induced by GGA1 overexpression accumulate Gag, whereas overexpressing any of the GGAs sequesters Arf1 (120, 121). Indeed, disrupting expression levels or activity of endogenous Arfs impaired Gag-membrane association and significantly inhibited retrovirus release, thereby implicating Arf proteins as critical cellular cofactors in Gag trafficking (120). The role of individual Arf members in HIV-1 Gag trafficking remains to be clearly defined and is the subject of ongoing investigation.

The host cell cytoskeleton, comprised of microtubules and actin filaments, has long been suspected of being involved in delivering viral proteins to particle assembly sites (57, 161). Microtubules mediate long-range cargo transport and hence are potentially suited for delivering viral components to assembly sites like the PM where actin filaments, which transport cargo across short distances, may play a role in virus budding and release. However, mechanistic details of how retroviruses exploit these cellular machineries during the late events of viral replication are largely lacking. Recent findings suggest that inhibition of actin and tubulin remodeling in T cells disrupts Gag and Env enrichment at polarized raft-like membrane domains, Env incorporation into virions, Gag release, viral infectivity, and cell-cell spread (113). Conversely, Gag expression has been reported to remodel the actin cytoskeleton at the site of particle budding (80). Two types of microtubule-based proteins with opposing motor activities exist; kinesins facilitate cargo transport toward the cell periphery, whereas dyneins promote cargo transport toward the perinuclear microtubule-organizing center (MTOC). The kinesin superfamily member KIF4 binds HIV-1 Gag (222), promotes Gag trafficking to the PM (146), and perturbing KIF4 function reportedly diminishes virus production. However, the mechanistic details of these observations are unresolved. Additional kinesins like KIF3C (20) and KIF3A (11) have also been implicated in late events of HIV-1 replication and are part of the kinesin-2 complex that mediates the movement of late endosomes/lysosomes to the PM (23). As discussed earlier, in addition to the PM-localized generation of virus particles, recent findings point to the existence of an alternative pathway that is dependent on endosomal trafficking of vRNA and Gag (137,

154). A role for dynein motor function in this intracellular transport pathway has been proposed (137).

Intracellular Ca^{2+} levels have been hypothesized to modulate HIV-1 Gag trafficking and assembly (88, 189). Annexin 2 (Anx2), a calcium-regulated protein, binds Gag (206) and is found in HIV-1 virions released from MDMs (35). The functional role of Anx2 in HIV-1 replication is currently unclear (195, 206), and the apparently cell-type-dependent role of Anx2 in modulating HIV-1 infectivity is yet to be mechanistically deciphered. Modulation of IP3R [inositol (1,4,5)-triphosphate receptor] function, i.e., gating of intracellular Ca^{2+} stores, was reported to influence Gag accumulation at the PM and virus release (54). What activates IP3R and how elevated Ca^{2+} enhances Gag accumulation at the PM remains to be determined. A cross talk between Ca^{2+} signaling and ubiquitination in HIV-1 biogenesis has also been postulated. RNAi-mediated depletion or inhibition of ubiquitination activity of a TGN-associated E3 ubiquitin ligase called POSH (plenty of SH3s) disrupted Gag localization at the PM, particle production, and virus infectivity (9). POSH's subsequently reported roles in modulating the activities of ESCRT-associated proteins (131, 231) and indirectly regulating cellular calcium homeostasis (226) may potentially account for its role in particle production and modulating virus infectivity. Interestingly, expression of SOCS1 (suppressor of cytokine signaling 1), a cellular protein shown to recruit E3 ubiquitin ligases and bind and stabilize microtubules, is induced during HIV-1 infection (205). SOCS1 binds Gag via the MA domain and enhances virus production when overexpressed in T cells, reportedly by promoting Gag ubiquitination and its subsequent association with microtubules, which together could enhance the stability and trafficking rate of Gag to the PM.

Recently, components of cytoplasmic structures such as P-bodies, which are cytoplasmic foci associated with mRNA turnover, and autophagosomes, which are intracellular vesicles that deliver cytoplasmic contents to lysosomes during the process of autophagy, have been implicated in HIV-1 particle production (47, 164). The P-body-associated Mov10 (Moloney leukemia virus 10 homolog) binds HIV-1 Gag via NC in an RNA-dependent manner (237), and is packaged into released virions (35). Modulating intracellular Mov10 levels affects particle production and virion infectivity (25, 72, 237). Further work is expected to clarify details of Mov10's role in HIV-1 replication. HIV-1 infection induces autophagy (42), disruption of basal autophagy impairs virion production (135), and autophagy-inducing rapamycin treatment causes a Nef-dependent enhancement of virus release (135). Nef's ability to block autophagosome maturation, thereby protecting viral proteins from degradation (135), and inhibit cell death in macrophages (175), has been hypothesized to contribute to the autophagy-induced increase in virus production. Ongoing research in this rapidly evolving field is expected to clarify the proposed role of autophagy in retroviral replication (47).

In several physiologically relevant cells types (T cells, macrophages, and dendritic cells), Gag has been observed to concentrate at points of cell-cell contact. These contact zones have been named virological synapses (59, 106, 116, 117, 148, 149, 158), by analogy with similar structures–immunological synapses–that form between antigen-presenting cells and T cells (86, 155). The HIV-1 virological synapse is characterized by polarization of the actin cytoskeleton and by accumulation of CD4, HIV-1 coreceptors (CXCR4 and CCR5), adhesion molecules, and tetraspanins, in addition to Gag and Env glycoproteins (111, 112, 114, 115). Because cell-cell transfer has long been known to be more efficient than cell-free infection (46), trafficking to the virological synapse has profound implications for HIV-1 replication in vivo. As discussed above, in macrophages, virus particles appear to assemble within an internal but surface-connected compartment (13, 45, 238). In dendritic cells, virus is thought to bind the cell surface and then be taken into a similar compartment (246). These

pre-assembled particles then move to the cell-cell junction following synapse formation (60, 85, 90, 148, 149, 234), allowing for efficient cell-cell transfer to take place. In T cells, Gag appears to traffic directly to the synapse where assembly may be favored (102, 141). The signals that regulate movement of Gag to the synapse remain to be defined, although it is noteworthy that synapses are raft-rich (115) and that depletion of cholesterol or disruption of the cytoskeleton interferes with Gag localization at the synapse (113, 115). It is clear that Gag trafficking and the site of virus assembly can be influenced by cell type and cell-cell interactions, and may also be modulated by cell density and other variables. Such variables may also influence the role of host factors in Gag trafficking and virus assembly. These considerations may in part explain some of the discrepancies observed among the different studies discussed above.

Role of the Viral RNA Export Pathway in Gag Trafficking and Assembly

Recent findings suggest that the mechanism by which vRNA traffics out of the nucleus into the cytoplasm may influence the Gag trafficking itinerary and modulate the efficiency of assembly. The lack of pre-mRNA splicing of vRNA precludes the recruitment of mRNA export adapters. Instead, the virus uses one of its accessory proteins, Rev, which binds a sequence in the vRNA called the Rev response element (RRE) and the cellular nuclear export receptor Crm1, to enable the nuclear export of vRNA (62, 63, 144, 151, 192, 245). Replacement of the HIV-1 RRE with the hepatitis B virus posttranscriptional regulatory element (PRE) yielded comparable levels of Gag protein expression but diminished budding efficiency by 10-fold (109). Intriguingly, this defect in virus production, attributed to altered Gag localization (exclusion from membrane rafts), Gag assembly site (intracellular instead of cell surface), and Gag assembly kinetics (slower), could be rescued by replacing the Gag MA domain with foreign membrane-targeting sequences (110). Hence, MA appears to disrupt the membrane localization and assembly of Gag if it is synthesized from vRNA that is trafficked out of nucleus via a Rev-independent mechanism. Remarkably, the role of the vRNA export pathway on assembly also appears to be cell-type and species-dependent. For instance, Gag expressed in certain rodent cell lines is assembly-incompetent. However, replacing the vRNA RRE with the constitutive transport element (CTE) from Mason-Pfizer monkey virus, which directly interacts with the NXF1 nuclear export factor, relieves this assembly block (219). The Gag translated from the CTE-harboring vRNA displays a modified localization pattern in mouse cells, i.e., PM association instead of cytoplasmic, which coincides with virion production. Interestingly, this phenomenon can be recapitulated even with Rev-/RRE-dependent vRNA if a regulatory sequence in the MA domain that keeps the NH_2-terminal myristyl group sequestered is deleted (215). This modification exposes the sequestered myristyl group and apparently causes Gag to be constitutively membrane associated.

Different hypotheses have been proposed to explain the apparent influence of the vRNA export pathway on MA-directed Gag targeting and assembly. Novel insights came from the characterization of a Gag mutant with amino acid substitutions in the MA HBR (36), which has been suggested to interact with vRNA (7, 96, 193). An HBR Gag mutant binds PI(4,5)P2-deficient liposomes with high affinity, localizes to intracellular sites, and displays enhanced virus release. Conversely, vRNA-bound WT Gag displays diminished association with PI(4,5)P2-deficient liposomes in an NC-independent manner (36). Hence, the MA HBR and the associated vRNA appear to prevent WT Gag from binding to PI(4,5)P2-deficient membrane, probably via myristate sequestration. Chukkapalli et al. (36) hypothesized that myristate sequestration and RNA binding may prevent Gag from associating with membranes until it reaches the PM, where the MA HBR binds PI(4,5)P2, thereby displacing the bound vRNA, triggering myristate exposure and stabilizing Gag

binding to the PM. The displaced vRNA could then act as a scaffold for NC-dependent Gag multimerization and particle assembly.

HIV-1 Budding and Release

As previously mentioned, HIV-1 usurps the ESCRT machinery to exit the cell, and failure to do so induces retention of immature virus particles whose membrane is continuous with that of the host PM (FIGURE 5). The ESCRT machinery is also implicated in MVB biogenesis, cytokinesis, and macroautophagy (94, 147, 204, 208, 209). The core of the ESCRT apparatus consists of four multiprotein complexes, ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, and several ESCRT-accessory factors including Alix and the AAA ATPase Vps4 (104, 241). Recent in vitro studies with giant unilamellar vesicles suggest that ESCRT-I and -II are key to bud formation, whereas ESCRT-III is the major player in mediating membrane scission (105, 240). Vps4 in turn is required in cells for the recycling of ESCRT machinery. HIV-1 recruits the ESCRT machinery via two independent mechanisms. The HIV-1 p6 domain harbors two late domains: the dominant late domain consists of the peptide sequence Pro-Thr/Ser-Ala-Pro [PT/SAP] that binds to the ESCRT-1 component Tsg101; the second late domain, Tyr-Pro-Xn-Leu (YPXnL, where X is any amino acid and n = 1–3 residues), binds to the adaptor protein Alix (15, 43, 67, 157, 228). Subsequently, Tsg101 and Alix recruit and activate ESCRT-III and Vps4. Perturbing Tsg101 function by siRNA-mediated depletion (75) or by overexpressing a dominant-negative version of Tsg101 (44) blocks virus release, essentially recapitulating the phenotype of PT/SAP deletion (83, 100). Conversely, fusing Tsg101 to a PTAP-defective Gag rescues the budding block imposed by PTAP deletion (145). HIV-1 thus appears to predominantly employ the PT/SAP-Tsg101 pathway to recruit the ESCRT machinery, and the role of Alix-mediated ESCRT-III recruitment is unresolved (71). It is now generally agreed on that membrane scission is mediated by the formation of concentric rings of ESCRT-III components that constrict and ultimately sever the bud neck (105). Despite a general mechanistic understanding of how ESCRT machinery promotes membrane budding and fission events, many details await further clarification. Advances in understanding physiological ESCRT-mediated processes should shed more light on how HIV-1 has successfully parasitized this cellular apparatus. One unresolved question relates to the role of Gag ubiquitylation in HIV-1 budding and release. The ESCRT-mediated recruitment of many cargo proteins into the MVB pathway requires ubiquitin modification of the cytosolic domains of the cargo (196), and Gag is ubiquitylated (84, 185). It remains unclear, however, to what extent Gag ubiquitylation promotes HIV-1 budding and release.

Recent findings from cryoelectron tomography (cET) analysis of immature and mature HIV-1 particles have considerably advanced our understanding of HIV-1 morphogenesis. Interesting revelations include observations that the Gag shell in immature virus particles is incomplete but is formed by a single, continuous hexameric lattice possessing an inherent curvature that is dictated by incorporation of irregular defects, and ESCRT participation in HIV-1 release is initiated early in bud formation (21, 30, 242). More recently, analysis of HIV-1 assembly and release from intact human cells by cET revealed similar organization of the Gag shell at budding sites and in released immature virus particles (30). Hence, it appears that structural organization of the released immature HIV-1 particle is pre-determined at cellular assembly sites.

Recent findings revealed host cell-mediated mechanisms that “restrict” the release of completely budded, mature virions from the cell surface. It has been known for some time that HIV-1 virions produced in the absence of the viral accessory protein Vpu remain tethered to the producer cell surface (82, 132, 223). This phenotype is distinct from that caused by late-domain mutations because, in the absence of Vpu, virions are mature, virion

and host cell membranes are discontinuous, and protease treatment can trigger virus release (165). A cellular protein, previously known as BST-2, CD317, or HM1.24, was identified as the causative agent of this tethering phenomenon and was renamed “tetherin” (167, 229). Tetherin expression is induced by interferon treatment (166), suggesting that it is a component of the cellular innate antiviral defense machinery. In addition to restricting the release and spread of infectious HIV-1, BST-2/tetherin exhibits comparable virion tethering activity against numerous enveloped viruses (125, 128, 194, 210). Like HIV-1, other tetherin-restricted viruses also encode anti-tetherin factors that apparently function via distinct mechanisms. Currently, BST-2/tetherin is thought to manifest its restriction activity by simply cross linking the viral membrane and the host cell membrane, thereby retaining the virions on the cell surface (212, 243). Remarkably, an “artificial” tetherin that lacks sequence homology to bona fide tetherin but retains its structural features restricts HIV-1 release (188). Hence, it appears that BST-2/tetherin function is independent of any other cellular cofactors. How Vpu antagonizes BST-2/tetherin action is currently under intense investigation (55). Vpu is believed to antagonize tetherin’s antiviral activity via two distinct mechanisms (52, 55, 91). Vpu promotes tetherin degradation, at least in part by utilizing the same host ubiquitin machinery that it employs to target CD4 for proteasomal degradation. However, whether Vpu-mediated degradation of tetherin is primarily proteasomal or lysosomal remains unresolved. Meanwhile, recent observations also point to the existence of an alternative Vpu-mediated anti-tetherin mechanism whereby Vpu promotes sequestration of tetherin in intracellular compartments (10, 52). Thus there appears to be both degradative and non-degradative mechanisms for Vpu-induced counteraction of tetherin.

Summary and Concluding Remarks

As discussed above, recent findings have considerably improved our general understanding of the viral and cellular determinants involved in the late events of HIV-1 biogenesis. Several factors that contributed to this progress stand out among many. The development of novel imaging techniques has dramatically improved the spatial and temporal resolution of in vivo observations, and parallel advances in the understanding of host cell biology have provided the framework for the generation of testable hypotheses to explain experimental observations. Advances in structural biology, including cryo-electron tomography, have greatly increased the limit of resolution of complex retroviral structures. Examples that demonstrate the impact of these advances include *1*) the demonstration that the apparently intracellular compartments that accommodate HIV-1 assembly and release in macrophages represent specialized PM invaginations that are connected to the PM by a network of nanoscale tubuluar structures, *2*) the identification of the phospholipid PI(4,5)P2 and an expanding set of cellular proteins as cofactors in promoting Gag trafficking to the PM, *3*) the observation of particle movement across virological synapses in several cell types, *4*) the elucidation of the cellular ESCRT pathway and mechanisms by which HIV-1 and other retroviruses usurp this apparatus to exit the cell, and *5*) the finding that viral RNA and nuclear RNA export pathways profoundly influence the subsequent events in retroviral replication.

Despite this progress, significant gaps still exist in our knowledge of the HIV-1 assembly and release pathway. Pressing questions include:

1) What are the viral and cellular determinants that define the preference for a particular membrane interface? Determining the mechanistic basis of HIV-1 assembly at different membrane microdomains such as membrane rafts, tetraspanin-enriched microdomains, endosome-like domains, and virological synapses will clarify many of the contrasting observations reported in the literature.

2) When and where do the viral RNA and Env glycoproteins engage Gag during the virus assembly process? Although genomic vRNA and Env glycoproteins are dispensable for virus assembly and release, infectious particle production requires their incorporation. Moreover, recent findings suggest that Gag-vRNA association may also determine Gag localization to productive virus assembly sites.

3) How do HIV-1 Gag polyproteins reach their assembly site(s)? This is currently one of the least understood aspects of the HIV-1 assembly and release pathway. The apparent involvement of numerous cellular factors and machineries complicates deciphering the Gag trafficking itinerary. However, technical advancements and identification of potential cellular cofactors via RNAi screens and other methods are expected to produce significant breakthroughs in the coming years.

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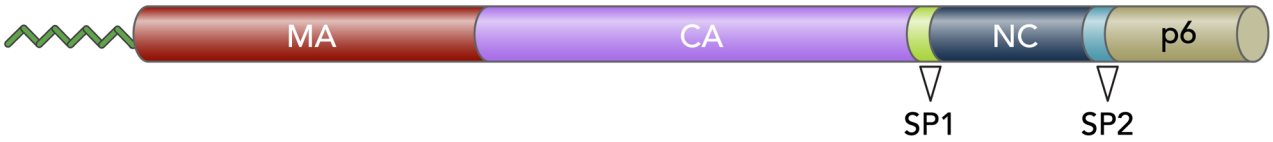

FIGURE 1. Schematic representation of the linear organization of the HIV-1 Gag polyprotein precursor Pr55Gag

The myristylated ( ) matrix (MA) domain, capsid (CA), nucleocapsid (NC), p6 domains, and the spacer peptides SP1 and SP2 are indicated.

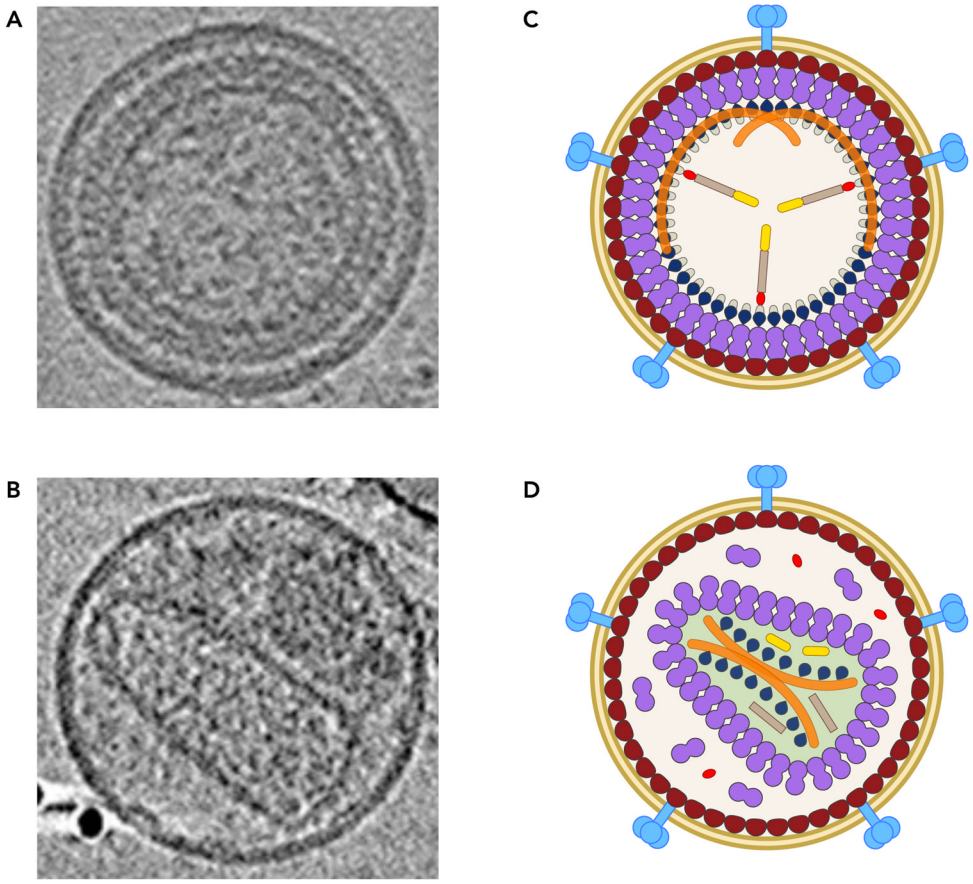

FIGURE 2. Cryoelectron micrographs and schematic representations of immature and mature HIV-1 particles

Central slices through cryo-EM tomograms of immature (*A*) and mature (*B*) virus particles that are ~130 nm in diameter (from Ref. 73). Schematic representation of immature (*C*) and mature (*D*) HIV-1 particles. Parts of figure were adapted from Ref. 73 and used with permission from Elsevier.

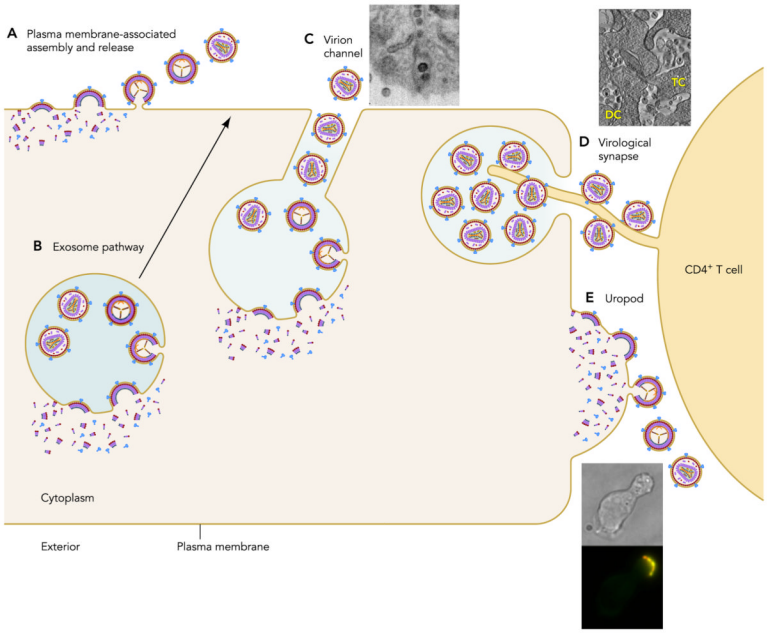

FIGURE 3. Schematic representation of the HIV-1 assembly and release associated with different membrane interfaces

A: as per the canonical view, HIV-1 Gag traffics to the inner (cytoplasmic) leaflet of the PM, independent of the cell type, where it initiates virus assembly and budding. *B*: an alternative proposal is that HIV-1 Gag assembles, in a cell-type-dependent manner, on the cytoplasmic face of intracellular vesicles such as LE/MVBs, and the ensuing virus particles bud into the intraluminal space. The virus-harboring vesicles then traffic to and fuse with the PM (e.g., exosome pathway), thereby resulting in the extracellular release of the virions. *C*: another model contends that such intracellular compartments in macrophages, where HIV-1 Gag assembles at and buds into, are connected to the PM via nanoscale tubules. The accompanying IA-SEM image shows the transverse section of a virion-containing channel in an HIV-1-infected macrophage (from Ref. 13). *D*: in another version of the cell-type-dependent HIV-1 assembly and release events, Gag trafficking and assembly are proposed to target virological synapses that promote efficient virus transmission. The accompanying IA-SEM image shows such a synapse, where filopodial extensions of a CD4+ T cell (TC) come in contact with HIV-1 virions that are sequestered within a network of PM-accessible compartments in a dendritic cell (DC) (from Ref. 60). *E*: in polarized T cells, HIV-1 Gag has been proposed to localize at the PM of uropod structures, which then become the favored contact sites between infected and uninfected T-cells. The accompanying epifluorescence microscope image shows the copatching (yellow) of Gag with the uropod-specific marker CD43 at the uropod of a T cell (brightfield image on *top*) (from Ref. 141). Parts of figure were adapted from Refs. 13, 60, and 141 and used with the authors' permission.

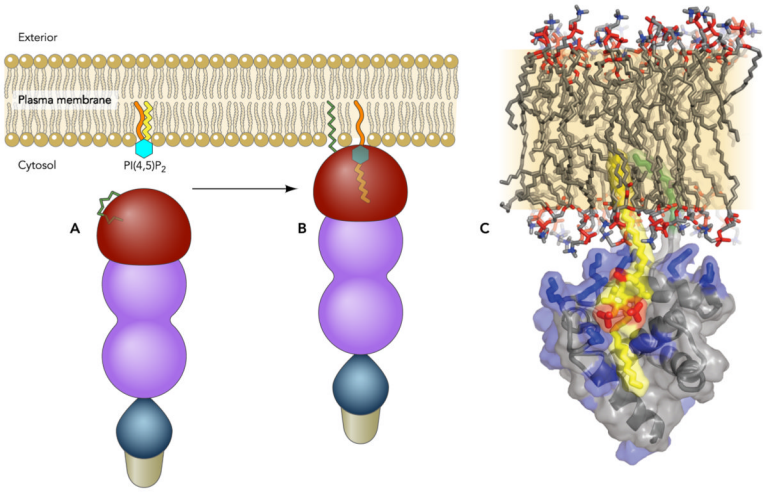

FIGURE 4. HIV-Gag association with PI(4,5)P2 at the PM

A: the NH2-terminal myristic acid moiety (green) of MA is depicted in its sequestered conformation PI(4,5)P2 embedded in the inner leaflet of the PM lipid bilayer is shown with its 1′ (orange) and 2′ (yellow) acyl chains in the lipid bilayer. *B*: binding between MA and PI(4,5)P2 leads to the flipping out of the myristate moiety into an exposed conformation and its subsequent insertion into the lipid bilayer, and, based on the model of Saad et al. (207), the extrusion of the 2′ acyl chain from the lipid bilayer and its sequestration by MA. *C*: according to Saad et al. (207), during the formation of the complex between HIV-1 MA and PI(4,5)P2, the 2′-unsaturated acyl chain of PI(4,5)P2 (yellow) binds to the hydrophobic cleft in MA and the myristyl group (green) of MA inserts into the lipid bilayer (image generously provided by Dr. M. Summers).

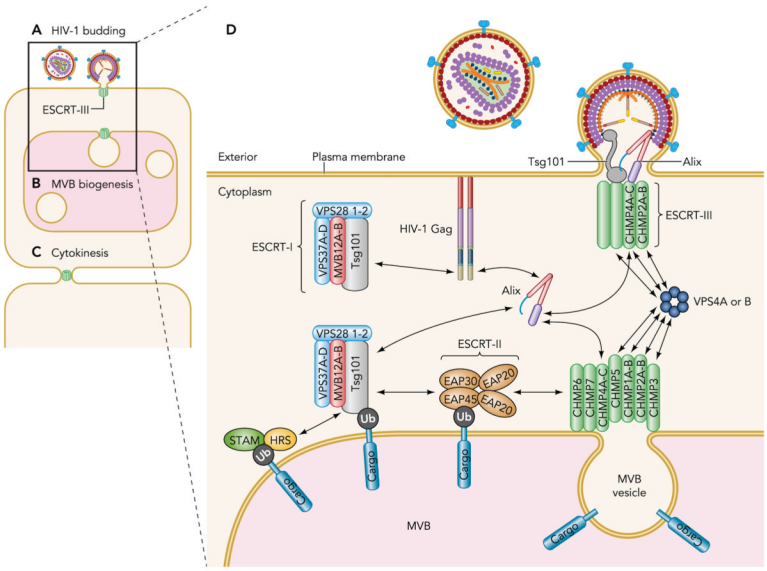

FIGURE 5.

Schematic representation of topological equivalence of HIV-1 budding and release (*A*), MVB biogenesis (*B*), and cytokinesis (*C*). *D*: the ESCRT machinery components implicated in HIV-1 budding and release. *D* was adapted from Ref. 71 and used with permission from Nature Publishing group; for details, see text and Ref 71.