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*Review Article*

# HIV Assembly and Budding: $Ca^{2+}$ Signaling and Non-ESCRT Proteins Set the Stage

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More than a decade has elapsed since the link between the endosomal sorting complex required for transport (ESCRT) machinery and HIV-1 protein trafficking and budding was first identified. L domains in HIV-1 Gag mediate recruitment of ESCRT which function in bud abscission releasing the viral particle from the host cell. Beyond virus budding, the ESCRT machinery is also involved in the endocytic pathway, cytokinesis, and autophagy. In the past few years, the number of non-ESCRT host proteins shown to be required in the assembly process has also grown. In this paper, we highlight the role of recently identified cellular factors that link ESCRT machinery to calcium signaling machinery and we suggest that this liaison contributes to setting the stage for productive ESCRT recruitment and mediation of abscission. Parallel paradigms for non-ESCRT roles in virus budding and cytokinesis will be discussed.

## 1. Focus of This Paper

Determinants intrinsic to the structural precursor polyprotein (Gag) that is encoded by the Human Immunodeficiency Virus-type 1 (HIV-1) and other retroviruses direct targeting of Gag to the plasma membrane, membrane and genome RNA binding, Gag multimerization, and budding of the assemblage into the extracellular space as virus particles (reviewed in [1–4]). Through a proteomic search aimed at identification of cellular factors that might participate with Gag and ESCRT, we identified the inositol 1,4,5-triphosphate receptor (IP3R) as a protein enriched in an endosome- and plasma-membrane-enriched fraction [5] only when Gag was expressed (unpublished observation). IP3R protein forms a transmembrane calcium ion ($Ca^{2+}$) channel that is mostly found on the membrane of the endoplasmic reticulum (ER), the major intracellular $Ca^{2+}$ store in the cell. IP3R has also been detected on the plasma membrane, late endosome/multivesicular bodies (LE/MVBs), and the nucleus (reviewed in [6–8]). Efficient HIV-1 Gag trafficking and viral particle release were shown to require activation of IP3R [9]. IP3R activation requires phospholipase-C- (PLC-) catalyzed hydrolysis of $PI(4,5)P_2$ to generate inositol 1,4,5-triphosphate (IP3), the activating ligand for the receptor (reviewed in [6–8]). Binding of IP3 initiates conformational changes leading to channel opening and release of $Ca^{2+}$ into the cytosol [10]. Earlier studies on HIV particle production had demonstrated that induction of a transient rise in the cytosolic $Ca^{2+}$ concentration resulted in a dramatic rise in viral particle release, suggesting that $Ca^{2+}$ is a limiting factor in late-stage replication [11, 12]. Taken together, these observations collectively suggested that IP3R is the physiological provider of the required $Ca^{2+}$. The proteomic search also identified several additional proteins that function in regulation of $Ca^{2+}$ signaling, including Sprouty2 (Spry2), a modulator of $Ca^{2+}$ signaling [13] and other modes of signaling [14, 15]. We demonstrated that Spry2 is also required for productive HIV egress [16, 17]. Proteins such as IP3R and Spry2 have been shown to function with the same elements of cytoskeletal and vesicular transport that are integral to ESCRT machinery [18–20]. Over the past few years, a number of other non-ESCRT host proteins have been shown to be required for Gag assembly. Some of these have been discussed in recent reviews [2, 21, 22]. We will discuss

how these host proteins set the stage for ESCRT recruitment and ESCRT-mediated abscission events. We apologize to those investigators whose studies may be pertinent but were not explicitly cited.

## 2. Introduction

Enveloped viruses, like HIV-1, exit the host cell by budding. The segment of the plasma membrane that serves as assembly platform evaginates to form the budded particle and becomes the viral envelope. Since the Gag precursor is the viral gene product that plays the key role in recruiting other viral components to the assembly site [23, 24], the assembly process must necessarily include a mechanism for stable localization of Gag at the plasma membrane (PM). Once on the PM, Gag has intrinsic assembly capability that is attributed to functions of its four domains (matrix-capsid-nucleocapsid-p6). The N-terminal matrix (MA) domain mediates membrane binding ([25–29] and references in [1]). The capsid (CA) domain provides Gag with capability for self-assembly into higher-order multimers ([30–35] and references in [36]). The nucleocapsid domain (NC) mediates binding to viral RNA and nonspecific RNAs as well as promoting Gag association [37–39] and references in [40]. The C-terminally located p6 region mediates the untethering of the assembled Gag particle from the host [41, 42]. Orderly cleavage of Gag at interdomain junctions within the structural precursor polyprotein by a virus-encoded proteinase [43–47] occurring concurrently with budding results in mature proteins whose rearrangement transforms the bud to a mature, infectious particle [48, 49]. The final step of the virus assembly process, which results in the pinching off of the particle from the host cell, is mediated by ESCRT proteins that have been recruited to the bud neck by motifs in p6 that are designated as "late" or L domains (reviewed in and references in [50, 51]). Thus, Gag is both necessary and sufficient for viral particle assembly [52].

## 3. Plasma Membrane Targeting: Role of PI(4,5)$P_2$

As a cytosolic protein, the synthesis of Gag takes place on soluble polysomes in the cell interior [53]. A myristoylation reaction occurs cotranslationally during which Gag acquires a myristoyl moiety on the N-terminal glycine which plays a role in assembly [28, 54, 55]. At the earliest experimentally feasible time points, Gag has been demonstrated to have a cytosolic distribution when examined by confocal microscopy [11], biochemical fractionation [56], and immunogold electron microscopy [57]. Eventually, the entire Gag population becomes membrane associated with the PM as the preferred site at steady state (references in [23]). This is consistent with the results of *in vitro* binding studies wherein MA, which is highly basic ([25–29] and references in [1]), mediates binding to membranes reconstituted with acidic phospholipids ([26, 27] and references in [1]). It is also consistent with observations that the cytoplasmic leaflet of the PM is unique among cell membranes in having a net negative charge due to high levels of acidic phospholipids [58]. The targeting phospholipid was identified as the complex acidic phospholipid, phosphatidylinositol 4,5 bisphosphate (PI(4,5)$P_2$) [59]. Depletion of PI(4,5)$P_2$, using plasmamembrane-targeted lipid phosphatases, caused Gag to be localized to LE/MVBs and prevented Gag localization to the PM [59]. PI(4,5)$P_2$ is mostly found on the PM where it represents a minor plasma membrane lipid component [60]. Structural analysis of PI(4,5)$P_2$ binding to HIV-1 MA shows contacts made by the head group (i.e., phosphates and inositol ring) with basic residues and the nestling of adjacent acyl groups into a hydrophobic cleft [61] while studies with full-length Gag underscored the importance of the phosphoinositide acyl chain [62]. These *in vitro* studies also predict initiation of Gag structural changes following PI(4,5)$P_2$ binding. Studies with the matrix protein show that PI(4,5)$P_2$ binding results in exposure of the N-terminal myristate [61]. Studies with Gag in the presence of nucleic acid reveal an interplay between binding to PI(4,5)$P_2$, binding to nucleic acid, and capsid (CA) domain-mediated self-association [63]. The model of Gag membrane association founded on Gag interaction with PI(4,5)$P_2$ is supported by the inhibitory effect on Gag particle release of depletion of plasma membrane PI(4,5)$P_2$ [59, 64, 65]. It should be noted that as important as PI(4,5)$P_2$ is to HIV-1 Gag membrane targeting, the importance of PI(4,5)$P_2$ to targeting and release of other retroviral Gags varies. Mo-MLV exhibits a preference and a requirement for PI(4,5)$P_2$ [66]. Equine infectious anemia virus (EIAV) budding is less impacted by depletion of PI(4,5)$P_2$ due to preferential binding to PI(3,5)$P_2$ [65]. PI(3,5)$P_2$ is a phospholipid that is predominantly associated with endosomal compartments at steady state [67] implying endosomal targeting of EAIV Gag in the cell. EIAV Gag trafficking requires such targeting as inactivation of the PI(3)$P_2$ 5-kinase, which is responsible for the endosomal placement of PI(3,5)$P_2$ [67], inhibits EIAV Gag VLP production [65]. ASV budding appears to rely on electrostatic interaction with acidic phospholipids and exhibits no specific reliance on phosphoinositide components of the PM [68]. Thus, HIV-1 Gag membrane association is mediated by a specific bipartite determinant in the MA domain comprised of myristate and basic amino acid clusters [1] with Gag-PI(4,5)$P_2$ binding serving as the basis for targeted membrane association. Gag's preferential association with the plasma membrane is due to two inherent features of PI(4,5)$P_2$: (i) the PM is where most of cellular PI(4,5)$P_2$ is located [60] and (ii) PI(4,5)$P_2$ molecules are products of *in situ* synthesis (i.e., PM-localized molecules are produced at the PM; [69]). Thus, PI(4,5)$P_2$ targeting provides a mechanism to direct Gag from its site of synthesis in the cell interior to the plasma membrane.

Detection of assembled HIV-1 Gag inside membrane compartments with the characteristics of LE/MVBs has been documented [70, 71], and altered Gag residency in LE/MVBs following stimulatory or inhibitory effects on virus production has been demonstrated [11, 72, 73]. Additionally, the virus particle has components that are typical exosome markers [74]. However, for macrophages, at least, those apparently intracellular membrane compartments

with LE/MVB features were demonstrated to be actually extracellular space delineated by intracytoplasmic plasma membrane [75, 76]. Moreover, Gag particle production has been shown to be insensitive to interference with LE/MVB function [77]. The role of the LE/MVB in Gag assembly and release thus remains controversial. We suspect that at the root of this controversy is the complex nature of the LE/MVB itself. It cannot be precluded that the endosomal machinery can interact with Gag in the traditional manner, wherein ESCRT machinery facilitates sorting of cargo proteins into MVBs for ultimate delivery to degradative compartments. However, the handling of sorted proteins by the MVB is not always unidirectional. Though targeted to the LE/MVB in both HeLa and Jurkat cells, the 29KE/31KE Gag mutant is released at near wild-type levels from Jurkat cells but is trapped inside HeLa cells [78] which shows that trafficking within the MVB can be influenced by its environment (i.e., cell dependence). EIAV Gag is another interesting case since, despite its endosomal targeting, EIAV Gag VLPs are released from cells such as COS-1 and HeLa [65]. It would be interesting to know if EIAV Gag induces any alteration in the MVB and, if so, whether this facilitates productive infection. Direct delivery of Gag to the site of release on the plasma membrane circumvents the potentially nonproductive outcome of Gag association with endosomal machinery. A Gag assembly model that incorporates Gag-$PI(4,5)P_2$-based targeting of Gag to assembly sites on the PM permits a more productive path from Gag synthesis to release of an assembled Gag particle.

## 4. Late Domains in Gag Recruit ESCRT Machinery

Budding structures accumulate on the plasma membrane if the C-terminal p6 region is missing from Gag [41, 42]. The p6 region bearing the L domain has counterparts in other retroviruses and is functionally exchangeable with these within and outside the genera; for example, the PTAP motif from the p6 region of HIV-1 Gag was shown to substitute for the PY motif in the L domain-bearing region (p2b) of the avian sarcoma virus (ASV) and vice versa [79–83] and references in [50, 51, 84, 85]. Functional exchangeability demonstrates that there are multiple, though not necessarily equally effective, ways for Gag to access the ESCRT machinery. Accordingly, Tsg101 as binding partner of the HIV PTAP motif and Nedd4 family members as binding partner of the ASV PY motif facilitate release of HIV-1 and ASV, respectively, through functionally exchangeable but independent routes (i.e., Tsg101 can replace Nedd4 function in facilitating ASV budding [86, 87]). Members of the Nedd4 family of ubiquitin ligases can also replace Tsg101 in facilitating HIV-1 release under certain circumstances [88–91]. The binding of the ESCRT adaptor, Alix, to the secondary L domain in Gag serves this purpose as well (reviewed in [92]). The ESCRT machinery is now known to comprise >25 proteins, organized into four complexes (ESCRT-0, -I, -II, and -III) that function sequentially along with several additional associated factors (reviewed in [93–95]). Irrespective of how Gag is linked to the ESCRT machinery, in all cases ESCRT-III and Vps4 must be recruited to the bud neck at the membrane site to execute the final bud scission event and to release the ESCRT factors from the assemblage for recycling back to the cytosolic pool for participation in future events [96, 97]. A feature of retroviral utilization of the ESCRT machinery is the selective use of the ESCRT complexes. HIV-1 viral particle production requires ESCRT-I and ESCRT-III but not ESCRT-II [98] while ASV requires ESCRT-II but not ESCRT-1 [99]. These observations, along with recognition that ESCRTs, which normally function in transport of some cellular proteins to degradative cellular compartments, are required for exit of assembled Gag from the cell, suggests that non-ESCRT host proteins may play a key role in allowing the ESCRT machinery to be utilized differentially by the virus compared to the host. Thus, non-ESCRT proteins may permit HIV to exploit ESCRT machinery by preventing the Gag-ESCRT complex from participating in interactions with ESCRT partners that are nonproductive for the virus.

## 5. Parallels between HIV-1 Budding, Cytokinesis, and Autophagy

*"All organisms do things the same way except that it is completely different in every detail" J. Haber*

The abscission event in virus budding results in separation of the enveloped virus from the host cell. Another process where the abscission event results in separation of two membrane-enclosed cellular entities is cytokinesis. Cytokinesis, itself a multistep process, is the terminal stage in cell division [100]. Abscission of the intercellular bridge/midbody results in separation of the mitotic daughter cells. Recruitment of ESCRT and mediation of the abscission event by ESCRT is the basis for the parallel between HIV-1 budding and cytokinesis [101, 102]. The parallel may extend to events occurring before ESCRT recruitment and participation, (i.e., in a pre-ESCRT stage). Paradigms that govern the pre-ESCRT stage of cytokinesis, which has been an active area of research long before discovery of HIV, may likewise apply to the pre-ESCRT stage of viral budding.

A theme that is emerging as a cell prepares for cytokinesis is the reshaping of calcium signaling [103]. Local and global elevations in cytosolic $Ca^{2+}$ level are achieved by ion release from the ER (the cell's major intracellular $Ca^{2+}$ store) and by influx from the extracellular environment [104]. Decrease in $Ca^{2+}$ content of the ER triggers activation of $Ca^{2+}$ influx channels on the plasma membrane and refilling of the ER store in a process called store-operated-calcium-entry (SOCE) [105, 106]. A major cellular change that occurs during cell division prior to cytokinesis is the uncoupling of $Ca^{2+}$ store depletion and SOCE [107, 108]. Why this is necessary is presently not known but the effect is to render the pre-ESCRT events in cytokinesis independent of SOCE and reliant on the internal stores as the $Ca^{2+}$ source. Independence from SOCE and reshaping of calcium signaling as a pre-ESCRT stage paradigm also appear to be the case for HIV-1 budding. Blockade of SOCE with 2-aminoethoxydiphenylborate (2-APB), a small molecule

inhibitor of store refilling through SOCE [109], had no effect on release of the HIV-1 Gag particle [110]. Blockade of a G protein-coupled receptor cascade [111] triggered by $Ca^{2+}$ entry through receptor-operated calcium entry (ROCE; [112]) also had no effect on Gag particle release [110]. Additionally, cells where productive Gag budding is occurring (i.e., expression of wild-type Gag) exhibit higher cytosolic $Ca^{2+}$ compared to mock-transfected cells or cells expressing a budding-impaired PTAP Gag mutant [110]. Possibly, insulating the calcium machinery from external $Ca^{2+}$ sources allows both virus budding and cytokinesis to proceed more efficiently. Figure 1 shows the elements of the $Ca^{2+}$ signaling machinery implicated in HIV-1 release.

Cytokinesis and viral budding share several general features (Figure 2). The first step in both processes is the targeting of the requisite components to the eventual scission site, that is, the plasma membrane. Formation of the cleavage furrow is a visual marker of initiation of cytokinesis and aspects of this event that appear similar to the budding process are furrow ingression, that is, a progressive narrowing of the eventual scission region to form a bud neck. In cytokinesis, the separating bodies are of comparable volumes; in viral budding, they are of unequal volumes. IP3R, intact $PI(4,5)P_2$, $PI(4,5)P_2$ hydrolysis, and $Ca^{2+}$ are all required for the normal progression of cytokinesis in cellular systems where cell division has been well studied, for example, spermatocyte and oocytes [113–116]. There is a requirement for $Ca^{2+}$ to maintain furrow or neck stability, necessitating constant PLC-mediated hydrolysis of $PI(4,5)P_2$ [117, 118]. Components involved in $Ca^{2+}$ mobilization and cytoskeleton remodeling are recruited to the furrow [117–119]. Similarly, in addition to intact $PI(4,5)P_2$ [59], HIV budding requires IP3R and PLC activity [9, 110]. Analogous to IP3R recruitment to the furrow in cytokinesis, there is also recruitment of IP3R to Gag budding sites on the plasma membrane [110].

In cytokinesis, the non-ESCRT protein mediating recruitment of ESCRTs is Cep55. Cep55 recruits Tsg101, a component of ESCRT-I, and Alix, an ESCRT adaptor protein that binds both ESCRT-1 and ESCRT-III, to the eventual scission site once furrow ingression is completed [101, 102, 122–124]. These ESCRT factors, in turn, recruit the ESCRT-III complex required to carry out the terminal step in cytokinesis, abscission, that is, the severing of the thin intercellular bridge that connects the two daughter cells [125–127]. The counterpart of the Cep55-ESCRT link in viral budding is the targeting of Gag to the eventual scission site on the plasma membrane and recruitment of Tsg101 and/or Alix through the L domains and eventually ESCRT-III.

Autophagy, the process involved in the breakdown of intracellular proteins and organelles, is now appreciated as a mechanism of great importance in both cell survival and cell death [128]. It is the latest cellular process linked to ESCRT function. Indeed, autophagy is a necessary postabscission step in cytokinesis [129]. Following cytokinesis, the dividing cells are connected by an intracellular bridge that contains the midbody. This structure persists long after division as a midbody derivative that is inherited asymmetrically by the daughter cell with the older centrosome. Recent findings in

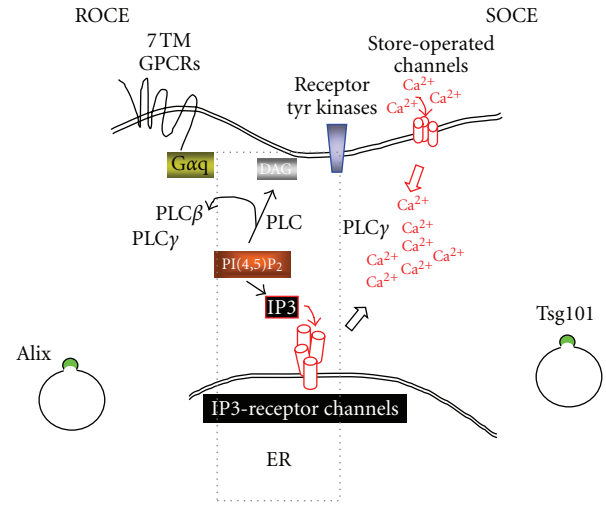


Figure 1: Elements of $Ca^{2+}$ signaling machinery implicated in HIV-1 release. Tsg101-mediated release requires the core elements, IP3R, $PI(4,5)P_2$, and PLC. Alix-mediated release requires these, SOCE and ROCE. It is not known whether SOCE and ROCE are controlled by distinct $Ca^{2+}$ channels [120] or if the same channel complexes mediate SOCE when recruited to lipid rafts and ROCE when they are outside of lipid rafts [121].

mammalian cells and in *Drosophila melanogaster* indicate that ESCRTs are required for efficient trafficking through the endolysosomal system where the autophagic cargo is degraded [130–132]. As with cytokinesis and viral budding, IP3R-mediated $Ca^{2+}$ signaling is emerging as critical for the pre-ESCRT stage in autophagy [133]. *De novo* synthesis of phospholipids is coupled with autophagosome formation [134]. Pairing phosphoinositides with $Ca^{2+}$ ions in endolysosomes has been suggested to control the direction and specificity of membrane trafficking [135]. All three processes, cytokinesis [136], viral budding [137], and autophagy [138, 139], require or involve SNAREs to conduct some of the critical events. The participation of calcium machinery components in all three processes suggests that the requirement for and reshaping of calcium signaling is a common feature governing their pre-ESCRT stages.

## 6. Non-ESCRT Proteins and Other Factors Engaged in the Pre-ESCRT Stages of HIV-1 Assembly

For a number of non-ESCRT host proteins shown to be important for release of the Gag particle [2, 4, 22, 140], disruption of the protein function does not result in the canonical L domain phenotype (i.e., arrested budding structures at the periphery of cells examined by EM). Rather, Gag is found in the cell interior. We and others [2] interpret this to indicate participation of these proteins in assembly step(s) preceding ESCRT-mediated budding. Some of these proteins have regulatory links to each other. Among these are the human vacuolar protein sorting (hVps) protein 18 (Vps18), a class C Vps complex component, and Mon2. Both have been shown to be required for Gag PM localization

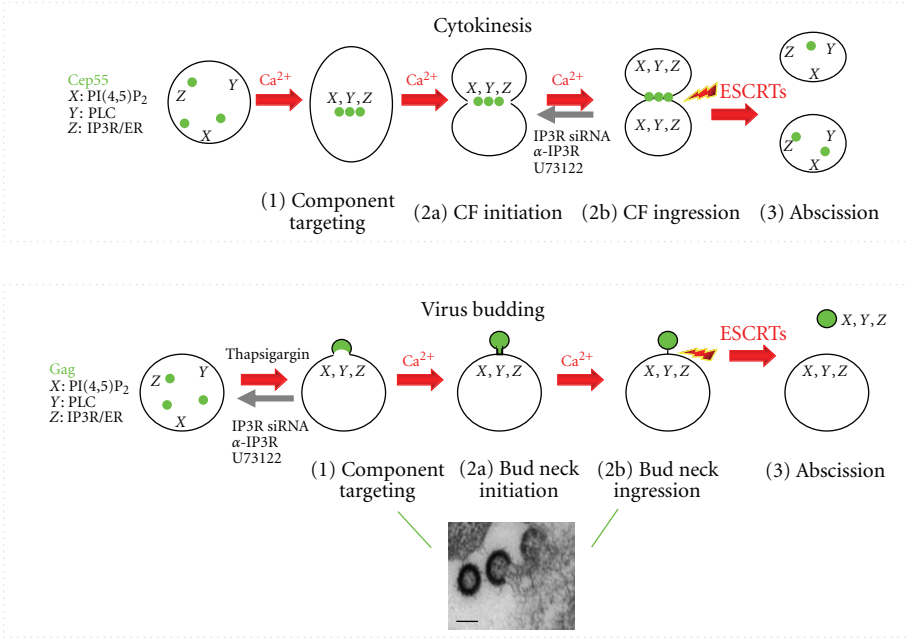


FIGURE 2: Similarities between cytokinesis (top) and viral particle production (bottom). CF: cleavage furrow. EM image shows HIV-1 VLPs in the process of budding. Bars indicate 100 nm.

and virus production [141]. In yeast, class C Vps proteins have been shown to regulate PM localization of at least one protein [142] and to assume roles antagonistic to ESCRT in the recycling of membrane proteins [143]. The human orthologue of Mon2 (hMon2) can bind and regulate the subcellular localization of adaptor proteins such as AP-1, AP-3, and Arf1 which have previously been shown to be required for Gag PM localization and Gag particle production [72, 144, 145]. The notion of non-ESCRT proteins regulating the activity of other non-ESCRT proteins in the pre-ESCRT stage has a parallel in cytokinesis as illustrated by the host protein, TEX14. This non-ESCRT protein binds Cep55 at the same motif used to recruit Tsg101 or Alix and negatively regulates ESCRT recruitment [146]. Through protein-protein interactions, non-ESCRT proteins could thus impose temporal and spatial control of the recruitment of participating proteins, including Gag itself, to assembly sites on the PM during the pre-ESCRT stage.

Another pre-ESCRT event is alteration of the lipid composition of the assembly site. Quantitative analyses indicate that the viral envelope differs from the PM of its host cell in having higher levels of cholesterol and $PI(4,5)P_2$ [58, 147]. Since the viral envelope is derived from the PM microdomain serving as the Gag assembly site, reorganization of the lipid bilayer in this location may occur as part of the assembly process. A feature of PM $PI(4,5)P_2$ is that the greater majority is sequestered by electrostatic interaction with basic proteins that are resident at the PM (e.g., myristylated alanine-rich C kinase substrate (MARCKS; growth-associated protein (GAP)43; N-methyl-D-aspartate (NMDA) receptor, and the epidermal growth factor receptor (EGFR)) and is only released by a local rise in $Ca^{2+}$ [148]. Another property of $PI(4,5)P_2$ is that it does not have a natural inclination for clustering due to the energy barrier posed by repulsion of the large polar head groups when they are in proximity. It has been shown that $Ca^{2+}$ can reduce this barrier and induce $PI(4,5)P_2$ clustering in lipid monolayers [149]. Recruitment of IP3R machinery to the cell periphery and release of $Ca^{2+}$ may function to increase the portion of PM $PI(4,5)P_2$ available for interaction with Gag and to permit the clustering of $PI(4,5)P_2$ molecules upon Gag multimerization. This model is summarized in Figure 3 and may explain how the budding requirement for both intact and hydrolyzed $PI(4,5)P_2$ could be simultaneously resolved.

That budding structures are still formed by Gag mutants with disrupted PTAP motifs despite their impairment in recruitment of Tsg101 or in cells where Tsg101 has been depleted [50, 51] indicates that assembly site membrane deformation is a pre-ESCRT stage event. Although not required for initiation [116], $Ca^{2+}$ is required for furrow ingression and for stability of the intercellular bridge in cytokinesis [113–116]. Furrow ingression in the presence of $Ca^{2+}$ leads to a productive ESCRT recruitment stage as indicated by completion of cytokinesis. Analogous to furrow ingression is the formation of the virus bud neck where the ESCRT scission complex is recruited. The fact that the budding structures of Gag mutants with disrupted PTAP motifs accumulate on the plasma membrane indicates a failure in ESCRT recruitment even though the mutant has been demonstrated to be capable of employing alternative modes of linking to ESCRT (i.e., via Nedd4 or Alix). Our study [110] shows that, in cells expressing HIV-1 Gag, IP3R was translocated from the cell interior to the periphery and colocalized with Gag on the plasma membrane. Interestingly, IP3R redistribution is not induced in cells expressing the PTAP Gag mutant even though release of the mutant, albeit

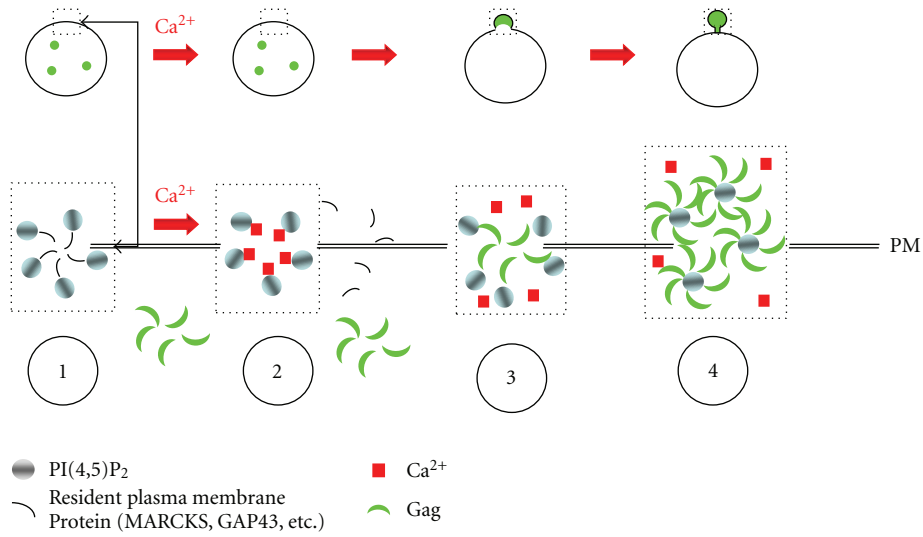


FIGURE 3: $Ca^{2+}$ facilitates Gag-$PI(4,5)P_2$ interaction and stabilization on the plasma membrane. Top, the squares highlight the top-down view of the plasma membrane shown below. Bottom, (1) most of the $PI(4,5)P_2$ on the plasma-membrane is sequestered with plasma membrane-resident proteins that are highly basic and therefore unavailable to Gag. (2) A local rise in $Ca^{2+}$ permits the cation to replace the resident proteins, freeing the $PI(4,5)P_2$ from these proteins. (3) $PI(4,5)P_2$, made available by $Ca^{2+}$, recruits Gag to the plasma membrane. (4) Gag multimerization forms local PIP2 clusters that stabilize Gag association with the membrane, preventing loss of Gag from the narrowing bud neck in preparation for ESCRT recruitment.

inefficient, also requires IP3R-regulated machinery. The lack of $Ca^{2+}$ store recruitment, which IP3R recruitment signifies, to the cell periphery of cells expressing such mutants indicates that, as is the case for furrow ingression, competency in linking to ESCRT is a property of bud necks formed in the presence of $Ca^{2+}$.

The ability of the endoplasmic reticulum to form tubules and small vesicles is what permits the stores to be recruited [150]. Movement of IP3R-contaning ER vesicles along microtubules has been shown to be facilitated by a kinesin [151]. Kinesins are a large family of cellular protein motors that use the energy of ATP hydrolysis to induce movement along the microtubule [152]. Kinesins have been identified as being involved in an intracellular process required for Gag release: (i) Kinesin KIF4 was reported to bind Gag directly through the MA domain [153] and was later found to regulate intracellular trafficking and stability of Gag [154]; (ii) Kinesin KIF3, a binding partner of AP-3 shown to be required for release of the viral particles assembled by Gag [72], has also been reported to be involved in Gag release [155]. Which particular kinesin is involved in IP3R transport is unknown. Kinesin-mediated translocation of IP3R along microtubules would allow for directed delivery of $Ca^{2+}$ stores to the budding site and, thereby, establish a localized region where $Ca^{2+}$ would be elevated. Thus, for $Ca^{2+}$ provision, utilization of the internal $Ca^{2+}$ stores may provide a major advantage over $Ca^{2+}$ influx which is mediated by channels that are homogenously distributed on the plasma membrane.

The notion that intact $PI(4,5)P_2$ is required for targeting Gag to the plasma membrane and that PLC-hydrolyzed $PI(4,5)P_2$ is required for ESCRT-recruitment-competent bud neck ingression suggests the need for regulatory mechanisms that would ensure availability of the right form of the phospholipid for the right event in the pre-ESCRT stage. The "hydrolysis stimulates synthesis" model proposes that hydrolysis and synthesis of $PI(4,5)P_2$ are tightly coupled events such that synthesis stimulates hydrolysis while $PI(4,5)P_2$ hydrolysis signals its production [69]. $Ca^{2+}$ might be a key regulator: $Ca^{2+}$ is an activator of the lipid kinase that is critical for $PI(4,5)P_2$ synthesis [156] and of the PLC that catalyzes $PI(4,5)P_2$ hydrolysis [157]. However, Gag PM targeting appears to require a more nuanced intact $PI(4,5)P_2$ population. Although it has been clearly demonstrated that depletion of $PI(4,5)P_2$ with plasmamembrane-targeted lipid phosphatases prevents Gag localization to the PM [59], other experimental approaches give different results. For example, increased Gag PM targeting and VLP release were not observed following a clear increase in PM $PI(4,5)P_2$ in cells treated with a PLC inhibitor [9]. Also, a loss of Gag PM targeting was reported in cells that did not exhibit a detectable change in $PI(4,5)P_2$ level or subcellular distribution [145]. There is growing recognition that PM $PI(4,5)P_2$ exists in multiple pools and that the dynamic nature of these pools is important for cellular processes mediated by $PI(4,5)P_2$ [148, 156]. Perhaps this conundrum, that is, the lack of a clear correlation between Gag PM targeting and the $PI(4,5)P_2$ level, reflects a requirement for a $PI(4,5)P_2$ pool that is specifically made available for Gag. The non-ESCRT proteins, Spry2 and ADP-ribosylation factor-1 (ARF1), have activities that make them potential participants in such regulatory mechanisms. Spry2 is required for Gag particle budding [16, 17] and for production of infectious virus (Ehrlich, Khan, Powell and Carter, unpublished observations). It has several activities that can affect $PI(4,5)P_2$ metabolism; namely, binding of phospholipase C [13] and of $PI(4,5)P_2$ [13, 17] and it can inhibit receptor-mediated

activation of PLC$\gamma$ [13]. Binding to PI(4,5)$P_2$ exerted the greatest influence on Gag particle production [17]. Involvement of ARF-1 in Gag assembly was demonstrated by Joshi et al. [145]. Although this protein is best known for its role in post-Golgi trafficking, ARF1 is also a stimulator of PI(4,5)$P_2$ synthesis by directly activating PI(4)P 5-kinase and by inducing formation of an enhancer of the kinase [158]. Thus, together with local $Ca^{2+}$, Sprouty and ARF1 proteins have the potential to ensure the dynamic existence of PI(4,5)$P_2$ pools specifically made available for interaction with Gag.

Several other non-ESCRT proteins whose dysfunction inhibited transport of Gag from the cell interior to the plasma membrane may also be involved in Gag assembly as pre-ESCRT stage participants. Admittedly, further studies will be needed to elucidate their exact contribution; however, interestingly, these proteins also have links to cytokinesis and autophagy. In addition to the aforementioned SNARES [145], these include citron kinase, a Rho effector [159]; Rab9 [160] and other GTPases [161]; POSH [162]; AP-1 [144]; NPC-1 [73]; and Filamin A [163]. Direct participation in cytokinesis is documented for citron kinase, AP-1, and Filamin A [164–166]. NPC-1 and POSH both affect the metabolism of two important factors in cytokinesis, cholesterol [167], and calcium [168], respectively. Rab9 and other small GTPases have been implicated in cytokinesis and autophagy [118, 169].

## 7. Non-ESCRT Proteins in the ESCRT Recruitment Stage

The formation of a Gag-Tsg101 complex occurs as part of the Gag assembly process as long as L domain-1 is intact. Although the precise stage at which Tsg101 docks on the PTAP motif is not known, association after stable bud neck formation might be more favorable as it precludes nonproductive interactions with ESCRT-II that would signal internalization of the Gag assemblage or premature ESCRT-III scission. Spry2 forms complexes with components of ESCRT-II [16]. Thus, Spry2 facilitates release driven by both the primary and the secondary HIV-1 Gag L domains, possibly due to its ability to compete with ESCRT-I factors for interaction with ESCRT-II components [16]. This notion is consistent with the fact that HIV-1 budding does not require ESCRT-II [98, 99]. Not surprisingly since the interaction of ESCRT-I with ESCRT-II leads to cargo internalization, it has been suggested that association with Tsg101 increases susceptibility to internalization [170]. Delaying the recruitment of ESCRT machinery to the budding site may provide a means of maximizing viral budding efficiency. A parallel to this as a regulation possibility in cytokinesis may be the aforementioned function of TEX14, a protein believed to control premature progression to the abscission stage by competing with Tsg101 and Alix for binding to Cep55 [146].

## 8. Concluding Remarks

In this paper, we have focused on proteins involved in steps in HIV-1 trafficking and budding that take place prior to Gag recruitment of ESCRT machinery. As described here, proteins that function in PI(4,5)$P_2$ binding, synthesis or hydrolysis, $Ca^{2+}$ store recruitment, IP3R-mediated $Ca^{2+}$ store release, and vesicular biogenesis or transport appear to comprise the major classes of participants in the pre-ESCRT stages. Cellular activities in almost all cells are regulated by common signaling systems and $Ca^{2+}$ is a ubiquitous intracellular messenger that is known to control a diverse range of processes. The discovery of $Ca^{2+}$ signaling as a cofactor in HIV-1 protein trafficking and release, its potential link to exploitation of the ESCRT machinery by the virus for viral particle production, and the general similarity of this coupling to other cellular activities in which ESCRTs participate, that is, cytokinesis and autophagy, may provide new therapeutic avenues for HIV treatment strategies.

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