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Review

The Role of Ubiquitin in Retroviral Egress

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HIV and many other enveloped viruses encode a late budding domain (L-domain) that recruits the cellular machinery that mediates the separation of the nascent virion from the infected cell. The ubiquitin–proteasome system has been implicated in the L-domain activity, but the exact role of ubiquitin transfer and ubiquitin-binding proteins in the last step of viral replication remains elusive. It is now widely accepted that the class E vacuolar protein sorting pathway mediates both viral budding and vesicle budding into the multivesicular bodies and, remarkably, both budding events share the same topology and similar requirements for ubiquitin. In this review, the role of ubiquitin in viral budding is discussed in the light of recent advances in the understanding of the cellular mechanisms that assist the last step of HIV-1 release.

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Gag proteins orchestrate assembly of retroviral particles by providing the structural components of virions and recruiting cellular cofactors that are essential to complete the formation and release of infectious particles from infected cells (1). The assembly process is mostly driven by activities encoded by the multiple domains of Gag, namely matrix (MA), capsid (CA), nucleocapsid (NC) and p6. Matrix interaction with the target membrane is primarily facilitated by the cotranslational myristylation of its N-terminal region (2) and this interaction is stabilized by a cluster of basic residues that bind to anionic phospholipids on the cytoplasmic face of the plasma membrane (3). The globular head domain of MA also regulates assembly by concealing the myristate at low Gag concentrations (4–6) and conferring co-operativity on Gag–membrane interactions (7). The formation of the budding virion is driven by lateral Gag–Gag interactions that are mediated mostly by CA and NC. The viral RNA also plays an active role in retroviral assembly by providing a scaffold that facilitates Gag:Gag interactions (8) and by encoding trafficking signals required to direct Gag to sites of viral assembly (9,10).

The last step of HIV-1 assembly is a membrane fission event mediated by the so called late budding domain (L-domain) at the C-terminal region of Gag, namely the p6 domain (11,12). In contrast to the other domains of Gag, p6 does not seem to play any structural role in HIV-1 virions but mostly serves as an adaptor domain that recruits cellular cofactors that mediate the separation of nascent virions from the infected cell (13). L-domain activity in p6gag is primarily mediated by a PT/SAP peptide motif (11,12) that is also found in other enveloped RNA viruses including Ebola virus, HIV-2, HTLV and Lassa fever virus (14–17). Additional viral L-domains present in HIV and other enveloped viruses are encoded by PPXY, LYPXL and FPIV amino acid motifs (18,19).

Class E Proteins and HIV-1 Budding

The identification of Tsg101 as the cellular cofactor that facilitates HIV-1 budding triggered a number of extraordinary advances in our understanding of the late steps of the retroviral life cycle (15,20–22). Importantly, budding of HIV-1 and other enveloped viruses is topologically identical to intra-lumenal vesicle formation in multivesicular bodies (MVBs) (Figure 1), a process driven by the class E vacuolar protein sorting (VPS) pathway, which includes a subset of cellular proteins that facilitate sorting of ubiquitinated receptors in transit to lysosomal degradation (23). This functional analogy between viral budding and MVB formation has been essential to illuminate the cellular mechanisms that facilitate the last step in retroviral assembly.

Tsg101 is the mammalian ortholog of a yeast protein termed Vps23 and is one subunit of a 350-kD complex (endosomal sorting complex required for transport-I, ESCRT-I), which also includes additional proteins, Vps28 and Vps37 (24). This complex recognizes ubiquitinated transmembrane proteins and delivers them to MVB vesicles (Figure 1). In yeast, the genetic ablation of ESCRT-I components, or any of approximately 18 so called class E VPS proteins results in failure to correctly sort ubiquitinated cargo and induces the formation of the class E compartment, an aberrant multilamelar prevacuolar endosome lacking intra-lumenal vesicles (25,26). Thus, Tsg101 appears to be required both for the budding of viruses that encode PTAP type L-domains (Figure 2) and the topologically equivalent process of vesicle budding into MVBs. A second

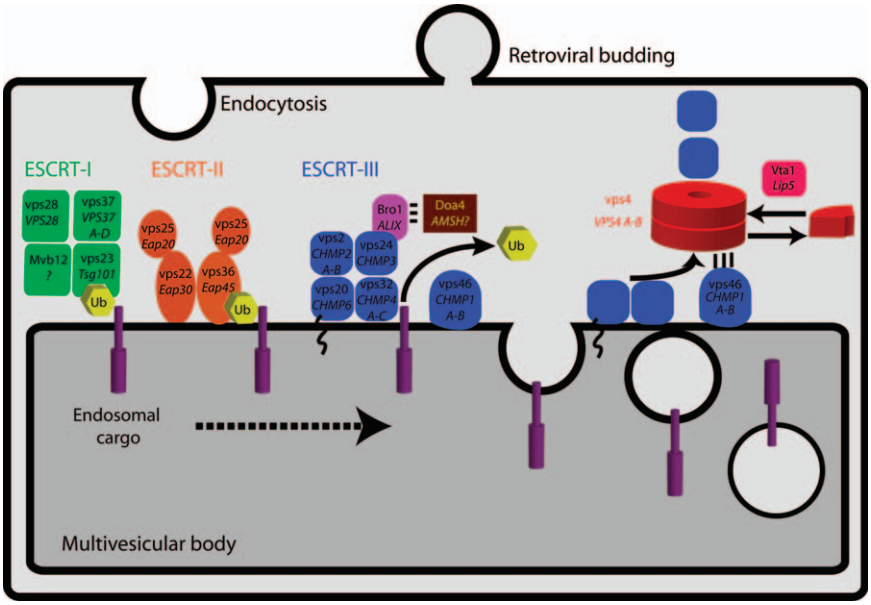

Figure 1: Conserved architecture of the class E VPS pathway. Components of the yeast class E pathway and proposed model for sorting of ubiquitinated cargo into the nascent vesicles of the MVBs. The nomenclature of the human homologues (in italics) follows the names of the yeast proteins. The figure also shows that the membrane topology of retroviral budding and MVB formation is equivalent, as opposed to endocytosis.

L-domain in HIV-1 Gag is a degenerate LYPXL (*LYP*LTS*L*) motif that binds AIP1/ALIX (Figure 2), the homologue of the yeast protein Bro1 (27). The LYPXL motif in HIV-1 Gag binds AIP1/ALIX with low affinity (28) and this interaction does not have independent L-domain activity but shows a synergistic activity with the PTAP/Tsg101 interaction to mediate full L-domain activity (29). In contrast, the LYPDL motif in EIAV binds AIP1/ALIX 60 times more tightly (28) and, in this context, provides a complete L-domain activity. Both retroviral LYPXL motifs bind to a conserved, hydrophobic groove on arm 2 of the V domain in AIP1/ALIX (28,30,31).

The crystal structure of the Tsg101–PTAP complex shows that the recognition of the PTAP motif by the Tsg101

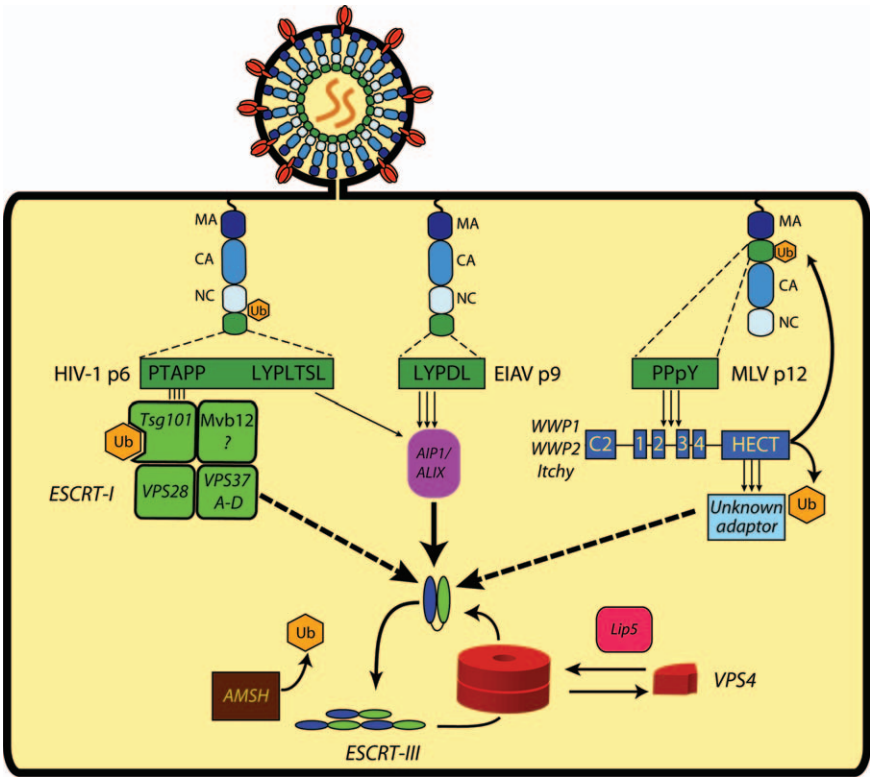

Figure 2: Cellular complexes and proteins involved in late domain activity. L-domains encoding different amino acid motifs enter the class E pathway through different adaptor proteins: PTAP motifs recruit ESCRT-I through the interaction with Tsg101, LYPXL motifs bind AIP/ALIX and PPXY motifs enter the pathway through the interaction with Nedd4-like ubiquitin ligases (WWP1, WWP2 and Itchy). The three types of L-domain require ESCRT-III and VPS4 to facilitate retroviral assembly. The dashed lines pointing to ESCRT-III represent unknown bridging factors.

ubiquitin E2 variant (UEV) domain is achieved primarily by two distinct pockets along the peptide-binding groove (32), suggesting that this interaction might be the target of novel therapeutic interventions. Initial studies also suggested that ubiquitination of HIV-1 p6 might play a secondary role in HIV-1 budding by increasing the affinity of Tsg101 binding by approximately 10-fold (20) and, according to this model, the Tsg101 UEV-ubiquitin (UEV–Ub) structure shows that the Tsg101 UEV domain can bind ubiquitin and PTAP peptides simultaneously (33). Alternatively, the UEV–Ub interaction might be required to recruit other ubiquitinated endosomal proteins.

It is becoming increasingly clear that a complete ESCRT-I is essential for PTAP-dependent viral budding (Figure 2). Functional studies show that transiently transfected VPS28 and VPS37 are relocalized to the sites of viral assembly by Gag (34,35) and that HIV-1 budding is not supported by Tsg101 mutants that do not bind VPS28 (35,36). Moreover, four human orthologs of yeast Vps37 have been recently identified (VPS37 A–D) (34,36,37) and RNAi experiments show that at least two of them (VPS37 B and C) are required for PTAP-mediated L-domain function (34), but the specific role of these proteins remains unclear. Mvb12, a fourth component of ESCRT-I, has been recently characterized in yeasts (38–40), but the identity of the human homologue and its role in viral budding remains unclear.

Current models of L-domain function are based on work in yeast that propose a sequential recruitment of the three ESCRTs (ESCRT-I,-II and -III) by the ubiquitinated endosomal cargo (Figure 1) (23). ESCRT-II is a Y-shaped complex (41–43) that can bridge ESCRT-I and ESCRT-III and the ubiquitin-binding activity of the complex has been ascribed to the Vps36/EAP45 protein by means of its N-terminal GRAM-like ubiquitin-binding in EAP45 domain (44,45). Based on the interactions of ESCRT-II with components of ESCRT-I and ESCRT-III, a role for ESCRT-II in HIV-1 budding was initially proposed (46,47). However, despite the overall conserved anatomy between the yeast and human complexes (47–49), ESCRT-II does not seem to be essential for retroviral budding (49,50), suggesting that there are alternative mechanisms to bridge ESCRT-I and ESCRT-III in the human class E pathway. AIP1/ALIX can also potentially connect ESCRT-I and ESCRT-III (27,47,49), but functional studies show that the L-domain activity of the PTAP motifs does not require AIP1/ALIX (49), thus suggesting that there are unknown factors bridging ESCRT-I and -III (Figure 2).

It is currently assumed that ESCRT-III encodes the core sorting machinery that forms a membrane-associated lattice and drives vesicle invagination and membrane fission during the MVB formation (51). ESCRT-III is composed of two functional subcomplexes, a membrane proximal complex (vps2 and vps20) that interacts with the endosomal membrane and a peripheral complex (vps24 and vps32) that recruits accessory proteins (52). A deubiquitinating activity encoded by Doa4 recycles ubiquitin from the endosomal cargo after ubiquitin-dependent commitment of the cargo into the forming vesicle of an MVB (Figure 1) (48,53,54). Once the luminal vesicle buds into the MVB, the AAA ATPase Vps4 is recruited by ESCRT-III to catalyze the disassembly of the ESCRT complexes for recycling and initiation of new rounds of sorting (55,56).

An essential role for ESCRT-III in retroviral budding was initially suggested by the inhibition of viral budding by a catalytically inactive form of VPS4 (20,35,57). Moreover, the overexpression of several ESCRT-III components (CHMP4A, CHMP4B, CHMP4C, CHMP2A, CHMP3 and CHMP5) also induces an arrest of viral budding that recapitulates the phenotype induced by mutation of the L-domain (27,47,49). More compelling evidence showing the role of ESCRT-III in retroviral budding has been recently presented by two papers (28,58), showing that mutations in ALIX that specifically abrogate the ALIX/CHMP4 interaction abolish the ability of ALIX to mediate budding. A role for CHMP1B/Vps46, an ESCRT-III-associated protein that coordinates recruitment of VPS4 and other ESCRT-III-binding proteins (59,60), is suggested by the specific inhibition of HIV-1 virion release by a dominant negative CHMP1B that lacks the C-terminal region of the protein (60).

A variety of observations indicate that every L-domain identified to date in enveloped viruses facilitate viral budding through a common mechanism. It is now well established that, for the most part, L-domains are interchangeable (61) and recent findings show that viral budding mediated by other L-domains share with HIV-1 the same requirements for ESCRT-III and VPS4 (20,27,35,47,49,57). In addition to the PTAP/Tsg101 interaction, it is now accepted that PPXY motifs recruit a subset of Nedd4-like HECT ubiquitin ligases (WWP1, WWP2 and Itchy) (14,62–64), whereas the LYPXL motifs recruit AIP1/ALIX to promote viral release (Figure 2) (27,47,49).

Ubiquitin and Viral Budding

The first indication of a potential role for ubiquitin in retroviral assembly came from the observation that there is an enrichment of unconjugated ubiquitin in ALV particles (65); this finding was subsequently confirmed in HIV, SIV and MLV virions (66) although the source of free ubiquitin in the particle remains unclear. Moreover, a small percentage of Gag is ubiquitinated (66), and the level of ubiquitination depends on the L-domain that is present (67,68), but the correlation between L-domain activity and Gag ubiquitination is unclear. It is well established that PPXY motifs induce Gag ubiquitination and facilitate viral egress by recruiting a subset of HECT ubiquitin ligases; however, the PPXY motifs do not present L-domain activity in the context of HIV-1 Gag despite the formation of ubiquitin–Gag conjugates that is comparable with that observed for MLV Gag (68). On the contrary, the ability of PTAP and

LYPXL motifs to promote viral release correlates with a decreased amount of ubiquitin conjugated to Gag (68,69), suggesting that L-domains recruit deubiquitinating enzymes. The interaction of the endosomal deubiquitinating enzyme AMSH (associated molecule with the SH3 domain of STAM) with the peripheral subunit of ESCRT-III (60,70,71) might account for the reduction of ubiquitin–Gag conjugates induced by active L-domains. Knockdown experiments suggest that AMSH is not essential for retroviral release, but the role of AMSH in the regulation of Gag ubiquitination is supported by the increased ubiquitination of Gag by a catalytically inactive AMSH (60). These results suggest that Gag deubiquitination might be a bystander effect of the recruitment of AMSH by ESCRT-III or, alternatively, that additional deubiquitinating enzymes interact with the class E pathway.

Initial studies on the effect of Gag ubiquitination in viral budding showed that lysine residues in close proximity of the HIV-1 and MLV L-domains are monoubiquitinated (66), but these lysines in p6 are not essential for L-domain activity (11,29,72,73). However, more detailed analysis of this region has shown that the cumulative mutation of the ubiquitin acceptor sites in the NC-p2-p6 region arrests budding at a late stage (74) and similar findings have been reported for *Rous sarcoma virus* (75). These results would be in agreement with indications of a functional cooperation of the NC-p2 region and the PTAP motif (76) and suggest that the ubiquitination of lysines in the proximity of the L-domain may facilitate the interaction of Gag with some of the ubiquitin-binding proteins of the class E pathway like Tsg101.

A role for ubiquitin in L-domain activity is also suggested by an inhibitory activity of proteasome inhibitors on viral budding that mimics the phenotype of an L-domain defective virus (67,77,78). A reduction of Gag ubiquitination as a consequence of the depletion of free ubiquitin in the cytoplasm has been proposed to explain the inhibition of L-domain activity by proteasome inhibitors (79). However, it is also possible that the relevant target for ubiquitination is a cellular protein, perhaps related to the class E VPS pathway or the endocytic machinery. An example that illustrates this possibility is given by the internalization of the growth hormone receptor, which is dependent on an intact ubiquitin conjugation system but does not require the direct ubiquitination of the receptor itself (80). A role for ubiquitin-binding proteins is supported by the dominant inhibition of viral budding by the mutation of the hydrophobic patch of ubiquitin that is required for endocytosis (76). Remarkably, the mutation of ubiquitin residues that are important for binding to Tsg101 also induce a dominant inhibition of HIV-1 release (33,76). Recent work shows that several ubiquitin-binding proteins are themselves regulated by monoubiquitination (81), including HRS, the upstream protein that recruits ESCRT-I to the endosomes (82,83). Consequently, it is possible that the inhibition of the proteasome may also inhibit the class E pathway and viral budding by altering the ubiquitination status of HRS or other ubiquitin-binding proteins in the pathway. In addition, inhibition of proteasomes might result in an inappropriate sequestration of ubiquitin-binding class E proteins (HRS, Tsg101, EAP45) as a result of the accumulation of polyubiquitinated proteins. In agreement with this idea, the L-domain activity of the LYPXL motif that is present in EIAV is not sensitive to proteasome inhibitors (84,85) perhaps because it bypasses ubiquitin-binding proteins by recruiting AIP1/ALIX, which is not thought to bind ubiquitin.

The Role of Ubiquitin Ligases on Viral Budding

The most compelling evidence supporting the role of ubiquitin ligases in viral budding comes from studies showing that several HECT ubiquitin ligases (WWP1, WWP2 and Itchy) act as a functional link between L-domains that contain PPXY motifs and the class E VPS pathway (14,62–64). A catalytically active HECT domain is required for optimal MLV release (64), but it remains unclear whether the relevant target of ubiquitination is a cellular cofactor or a structural component of the virus. Intriguingly, the HECT domain of WWP1 is sufficient to interact with the VPS4-induced class E compartment (64), but none of the known human class E components interact with WWP1, suggesting that unidentified factors bridge the HECT domain with the class E VPS pathway. In agreement with this concept, a WWP1 fragment lacking the HECT domain is a much more potent inhibitor of PPXY-mediated L-domain activity than a catalytically inactive WWP1 (64). Recent work in yeast indicates that sorting of Sna3 to MVBs shows some intriguing similarities with PPXY-mediated viral budding (86,87). Endosomal sorting of Sna3, a possible proton transporter, is mediated by a PPAY motif that interacts with Rsp5, another HECT ubiquitin ligase. Interestingly, ubiquitination of Sna3 itself is not essential for sorting, although Sna3 ubiquitination exhibits a more efficient sorting to the MVB. In contrast, the ubiquitin ligase activity of Rsp5 is essential for Sna3 MVB sorting, suggesting that ubiquitination by Rsp5 is required in a cargo ubiquitination-independent manner. Thus, it seems likely that HECT ubiquitin ligases interact with ESCRT-III by means of an as yet unidentified bridging factor that might be regulated by ubiquitination (Figure 2).

Another potential role of ubiquitination in viral budding is suggested by the interaction of Tsg101 with PTAP-containing E3 ubiquitin ligases, namely Tsg101-associated ligase (Tal) (88) and Mahogunin (89). Amit et al. propose a model whereby Tal inactivates Tsg101 through the multiple monoubiquitination of its C-terminal region, thus regulating the recycling of Tsg101 by shuttling between a membrane-bound active form and an inactive soluble form. However, it is also possible that the role of the Tal/Tsg101 interaction is to regulate the steady-state levels of Tsg101 through ubiquitin-dependent degradation of free

uncomplexed Tsg101 (our unpublished results). In contrast, Mahogunin induces multi-monoubiquitination of Tsg101 (89), which is likely to modulate Tsg101 activity rather than promoting proteasomal degradation. It is tempting to speculate that Tsg101 monoubiquitination might regulate binding to the other ESCRT-I components, although there is no experimental evidence supporting this model.

Recent work has involved POSH (plenty of SH3s), a *trans* Golgi network-associated ubiquitin ligase, in infectious HIV-1 production (90). The knockdown of POSH inhibits Gag transport to the plasma membrane, inducing a phenotype that is clearly unrelated to L-domain activity. A role of POSH in the ubiquitination of an unknown cellular target is suggested by the same levels of Gag ubiquitination in the presence or absence of POSH.

Concluding Remarks

The recent identification of the ESCRT proteins as components of the cellular pathway that mediates budding of many enveloped viruses has provided some plausible hypothesis to explain the mysterious role of ubiquitin in the last steps of HIV-1 replication. The fact that ubiquitin binds several proteins involved in viral budding suggest that ubiquitination of Gag and ESCRT proteins might contribute to the recruitment of the cellular machinery that mediates L-domain activity. Moreover, ubiquitin might also contribute to the regulation of the activity of the ESCRT proteins. In summary, despite providing important clues and inspiring the research in this area, the role of ubiquitin in retroviral budding is still a central question in the field without a definitive answer.

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