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# Late budding domains and host proteins in enveloped virus release

Paul D. Bieniasz

*Aaron Diamond AIDS Research Center and Laboratory of Retrovirology, The Rockefeller University, 455 First Avenue, New York, NY 10016, USA*

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## Introduction

A necessary step in the genesis of an extracellular enveloped virus particle is the formation of an outer virion membrane. Often, virion membrane is derived from host cell membranes, and this presents a problem because separation of virion from host membrane is not a trivial or spontaneous step. Indeed, viruses have evolved a number of strategies of varying complexity to deal with this issue. Herpes- and poxviruses for instance, have developed sequential membrane wrapping and fusion activities that ultimately give rise to extracellular virus particles with one or more lipid bilayers. For simpler viruses that acquire their envelopes by budding through a single host membrane bilayer, the fission of the nascent virion membrane from that of the host cell requires a different approach. At least some of these viruses have solved the problem by mimicking and/or coopting factors that cells usually employ during the formation of cytoplasm-containing vesicles within endosomes. The virus and cell encoded activities that are responsible for these virion and vesicle budding events are currently an area of intense interest and a number of interesting parallels and differences in the two processes are being uncovered.

## L-domain types and distribution

The first compelling evidence that the simple enveloped viruses might require specific virus or cell encoded activities in order to separate virion from cell membrane came from studies of HIV-1 mutants with curious assembly defects (Gottlinger et al., 1991). Specifically, HIV-1 Gag proteins carrying mutations in the carboxy-terminal p6 domain (Fig. 1A) were found to assemble into relatively normal immature virions, but these particles failed to separate from the host cell (Gottlinger et al., 1991). This late-assembly defect was later recapitulated in Rous sarcoma virus (RSV) wherein the defect mapped to the p2b domain of Gag (Wills et al., 1994; Xiang et al., 1996). Thereafter, numerous other retroviruses–essentially all that were examined–were shown to encode similar activities. The term late assembly or 'L'-domain was coined to describe motifs whose mutation induced the characteristic defective assembly phenotype whereby virion and cell membranes failed to separate. Examples of this phenotype for HIV-1 and murine leukemia virus (MLV) are shown in Fig. 1B.

A theme that emerged from these studies was that retroviral L-domains were one of three types, characterized by the presence of distinct core sequence motifs (Fig. 1A). The originally identified HIV-1 L-domain was encoded by a PTAP motif (Gottlinger et al., 1991; Huang et al., 1995), while that of RSV was shown to be a PPXY motif (Xiang et al., 1996).

*E-mail address:* pbienias@adarc.org.

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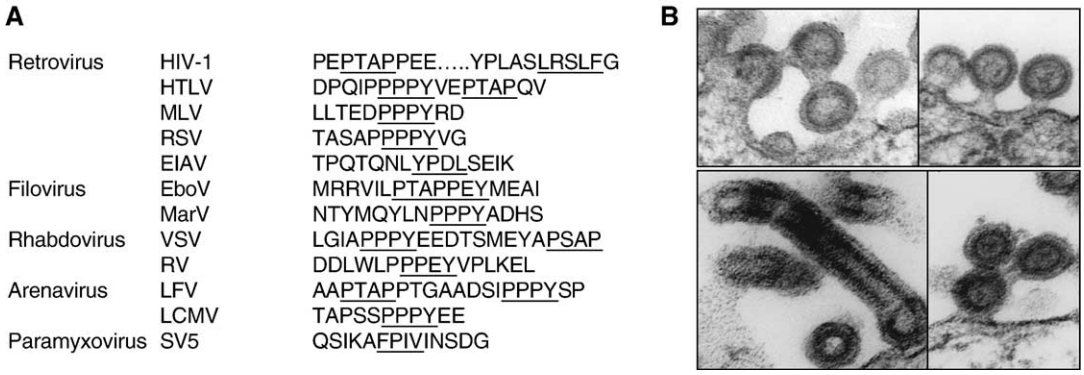


Fig. 1. Viral Latc budding or 'L'-domains. (A) Examples of L-domain motifs from diverse enveloped viruses are shown. Sometimes, a single recognizable motif is present, but frequently, two L-domain motifs are present within a short linear sequence. (B) Characteristic late budding defect resulting from inhibition of L-domain function in HIV-1 (top panels) or MLV bottom panels.

Similar motifs in numerous other retroviruses were shown to encode L-domain activity (Bouamr et al., 2003; Wang et al., 2002; Yasuda and Hunter, 1998; Yuan et al., 2000), while an L-domain encoded by equine infectious anemia virus had the sequence YPXL (Puffer et al., 1997). The identification of sequence motifs in retroviruses led to the prediction that other simple enveloped viruses that contained the same or similar motifs within their structural proteins should also be dependent on them for efficient budding. Indeed, this proved to be the case, and today we know that PTAP and/or PPXY motifs facilitate the budding of an array of retroviruses, filoviruses (Harty et al., 2000), rhabdoviruses (Craven et al., 1999; Harty et al., 1999) and arenaviruses (Perez et al., 2003) (Fig. 1A).

An important finding that emerged from early studies on L-domains was their ability to function as relatively autonomous units. In other words, they could promote virus release when transplanted into a different context and when placed in unnatural positions within a retroviral Gag protein (Parent et al., 1995). While more recent studies have indicated that the ability of L-domains to function in heterologous contexts is not universal (Martin-Serrano et al., 2004; Strack et al., 2002), these earlier findings proved extremely conceptually useful, in part because they considerably facilitated the identification of new L-domains. Indeed, the recent identification of a novel L-domain motif FPIV in paramyxoviruses (Schmitt et al., 2005) is based, in part, on the ability of the motif to functionally replace the PTAP motif in HIV-1 Gag. Moreover, the autonomous way in which L-domains appeared to function (Parent et al., 1995) suggested that they would act by recruiting other viral or cellular factors rather than directly affecting particle morphognenesis.

## Class E vacuolar protein sorting factors are required for L-domain activity

The notion that L-domains acted as docking sites for factors proved correct and cellular proteins that are recruited by each of the three prototype L-domains have been identified. PTAP motifs, for example, act by recruiting Tsg101 (Demirov et al., 2002; Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001). A variety of physiological activities had previously been ascribed to this protein, but its primary role is widely accepted to be in the determining the fate of transmembrane proteins at the limiting membranes of endosomes (see below). Evidence that Tsg101 recruitment is the mechanism by which PTAP L-domains facilitate virus budding is compelling: (1) there is an almost perfect correlation between the ability of HIV-1 p6 mutants to bind Tsg101 and to mediate virion production (Garrus et al., 2001; Martin-Serrano et al., 2001); (2) depletion of Tsg101 using small interfering RNA induces a characteristic L-domain mutant-like budding defect that is reversed by reintroduction of Tsg101 (Garrus et al., 2001); (3) artificially tethering Tsg101 to sites of HIV-1 particle assembly Gag reverses the late budding defect induced by PTAP mutation (Martin-Serrano et al., 2001); (4) expression of PTAP binding fragments of Tsg101 induces a late budding defect in HIV-1 (Demirov et al., 2002); (5) expression of either HIV-1 Gag or Ebola virus matrix protein in cells induces the recruitment of Tsg101 from intracellular locations to particle assembly sites at the plasma membrane (Martin-Serrano et al., 2001). Tsg101 contains several protein domains that are required for virus release. An N terminal ubiquitin E2 variant (UEV) domain binds PTAP motifs (Garrus et al., 2001; VerPlank et al., 2001) and structural analysis reveals the presence of a pocket in which PTAP binds that may be a viable target for pharmacologic intervention (Pornillos et al., 2002a, 2002b). A proline rich domain of unknown function links the UEV domain to coiled-coil and C-terminal domains that mediate interactions with other protein partners (Bache et al., 2004; Bishop and Woodman, 2001; Eastman et al., 2004; Martin-Serrano et al., 2003b; Stuchell et al., 2004), and induces Tsg101 multimerization (Martin-Serrano et al., 2003b).

Tsg101 is a member of a family of proteins whose orthologs in yeast were identified in screens for factors that are required for the sorting of proteins to the interior of the vacuole/lysosome (see Katzmann et al., 2002 and Pornillos et al., 2002a, b for review). In yeast, the Tsg101 ortholog is termed Vps23 and in addition to sorting defects, genetic ablation of this, or any one of approximately 18 or so other factors, results in the formation of a so-called 'class E' compartment (Katzmann et al., 2002). This is an aberrant enlarged endosome that is thought to arise because of a failure in inward invagination and budding of late endosomal membranes to

form multivesicular bodies (MVB). The discovery that Tsg101 was required for the release of certain enveloped viruses was especially intriguing because of obvious similarities between the process of viral budding and MVB vesicle budding (Pornillos et al., 2002a, 2002b). Indeed, the two processes can be viewed as fundamentally the same, in that both involve membrane invagination away from the cytoplasm and the pinching off of a vesicle, whose contents are cytoplasmic in origin, into a lumenal or extracellular milieu. Viral budding and vesicle budding differ only in that they occur at different sites in the cell, and the major contents of the resulting membrane enclosed unit are viral in one case and cellular in the other. Thus, it was intuitively satisfying that viral budding would exploit cellular factors normally required for vesicle budding and subsequent investigations have expanded on this notion. In yeast, a major subset of the class E factors participate in the formation of at least three major protein complexes, termed 'endosomal sorting complexes required for transport' (ESCRT) -I, -II and -III (Babst et al., 2002a, 2002b; Katzmann et al., 2001) (see Fig. 2). The anatomy of these complexes is largely preserved in mammals and most of the mammalian components of the class E VPS machinery could be identified by sequence homology to their yeast counterparts (Pornillos et al., 2002a, 2002b). Perhaps unsurprisingly, however, the mammalian class E VPS machinery has been slightly elaborated during eukaryotic evolution. Tsg101 is one subunit of mammalian ESCRT-I, which, in addition, includes two additional proteins VPS28 and VPS37. While the VPS28 binding property of Tsg101 could be shown to be required in order for it to mediate HIV-1 release (Martin-Serrano et al., 2003b; Stuchell et al., 2004), the identity of human VPS37 was not immediately obvious, given that no closely related homologue could be easily identified in human sequence databases. However, four different versions of VPS37 were recently identified by homology searching, and by using yeast two-hybrid screening with Tsg101 as a bait (Bache et al., 2004; Eastman et al., 2004; Stuchell et al., 2004). Two of these have been shown to be important for PTAP-dependent viral budding (Eastman et al., 2004). Thus, it appears that a complete ESCRT-I complex is recruited by PTAP motifs and required to induce virus release.

Two other protein complexes form important subcomponents of the mammalian class E VPS machinery. One complex, termed ESCRT-II comprises 3 proteins, EAP20, EAP30 and EAP45. A third complex is composed of so-called 'charged MVB proteins' (CHMPs). There are 6 CHMP-related proteins in yeast, some of which have been duplicated in mammals to make a total of 10 proteins. Several studies have documented protein:protein interactions between ESCRT-complex components and directed yeast two-hybrid screens have revealed rather extensive protein:protein interaction networks encompassing the ESCRT complex components and other class E VPS factors (Babst et al., 2002a, 2002b; Bowers et al., 2004; Martin-Serrano et al., 2003a, 2003b; Strack et al., 2003; von Schwedler et al., 2003) (Fig. 2). While there are some differences in details of these studies (particularly there are differences between yeast and mammalian systems), there is general concordance in descriptions of the mammalian class E VPS pathway (Martin-Serrano et al., 2003a; von Schwedler et al., 2003).

In addition to forming a complex with VPS28 and VPS37, Tsg101 also binds to two other mammalian class E VPS factors, AIP-1/ALIX and Hrs, orthologues of the yeast class E VPS factors, Bro1 and Vps27, respectively (Bache et al., 2003; Katzmann et al., 2003; Lu et al., 2003; Martin-Serrano et al., 2003a; Pornillos et al., 2003; Strack et al., 2003; von Schwedler et al., 2003). Like various viral structural proteins,

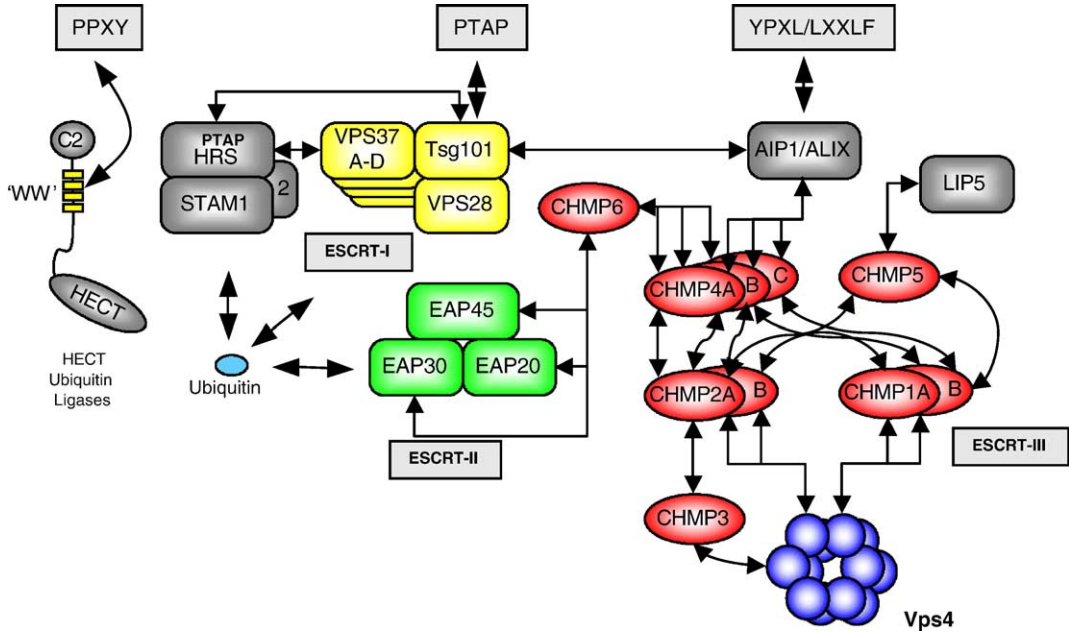


Fig. 2. Protein:protein interactions among components of the mammalian class E VPS pathway. Factors contributing to the complexes known as ESCRT-I, -II and -III are shown in yellow, green and red, respectively. There are minor differences in the precise nature of the interaction network in the literature pertaining to mammalian proteins and more extensive differences in the yeast counterpart (see text for details) but the overall anatomy of the network is largely agreed upon.

Hrs also contains a PT/SAP motif that contributes to its ability to bind to Tsg101 (Bache et al., 2003; Lu et al., 2003; Pornillos et al., 2003). In addition, there are PT/SAP independent interactions involving the C-terminal domain of Tsg101 and each of the four variants of VPS37 also binds Hrs (Bache et al., 2004; Eastman et al., 2004; Stuchell et al., 2004). Overall, there appears to be a particularly intimate association between Hrs and ESCRT-I. Because of this, and the fact that Hrs binds to phosphatidyl inositol-3-phosphate on endosomal membranes, Hrs may be the major factor that nucleates the recruitment of the class E VPS pathway to form a budding vesicle (Katzmann et al., 2002; Pornillos et al., 2002a, 2002b; Raiborg et al., 2003). While Hrs itself appears not to be required for viral budding (S. Eastman personal communication), an Hrs fragment can substitute for HIV-1 L-domain and mediate particle release when fused to the carboxy-terminus of HIV-1 Gag (Pornillos et al., 2003), presumably because of its ability to efficiently recruit ESCRT-I.

Another ESCRT-I binding protein, AIP-1/ALIX, plays a particularly important role in viral budding not only because it appears to act as one bridging factor between ESCRT-I and ESCRT-III, but also because it is bound directly by a second class of L-domains (Martin-Serrano et al., 2003a, 2003b; Strack et al., 2003; von Schwedler et al., 2003). Indeed, the incorporation of significant quantities of AIP-1/ALIX into primate lentivirus particles led to the discovery that they encode a second L-domain that conforms to the consensus LXXLF within p6, in addition to PT/SAP motif (Strack et al., 2003). In fact, the LXXLF motif carried by primate lentitiviruses and YPXL motifs carried by the equine lentivirus EIAV appear to be variants of the same class of L-domain, since both bind AIP-1/ALIX and both are dependent on it for activity (Martin-Serrano et al., 2003a, 2003b; Strack et al., 2003; von Schwedler et al., 2003).

The role of ESCRT-II in viral budding is less clear. Each of the ESCRT-II components EAP45, EAP30 and EAP20 binds to each other and to the ESCRT-III protein CHMP6 (Martin-Serrano et al., 2003a, 2003b; von Schwedler et al., 2003). Moreover, one of two studies that tested for interactions between ESCRT-I and ESCRT-II found that ESCRT-II proteins bound to Tsg101 (von Schwedler et al., 2003). While a structure has been solved for the ESCRT-II complex from yeast (Hierro et al., 2004; Teo et al., 2004), and ESCRT-II can bind to ubiquitin (Alam et al., 2004), whether and how this complex plays an essential role in vesicle and viral budding is unknown at present.

There is strong evidence that ESCRT-III plays an important role in viral budding, but the precise details of how this occurs are not well understood. The CHMP proteins that comprise ESCRT-III exhibit a significant degree of sequence homology to each other, and to corresponding yeast proteins, and show a substantial predicted propensity to form coiled-coils. Moreover, they exhibit a striking distribution of charged amino acid residues, with large clusters of basic and acidic amino acid residues situated toward their amino and carboxy termini, respectively (Howard et al., 2001). In addition, at least some CHMP proteins have affinity for membranes (Babst et al., 2002a), with CHMP6 carrying an amino-terminal myristoyl group. CHMP proteins also have extensive interactions with each other and with other class E VPS factors (Babst et al., 2002a; Martin-Serrano et al., 2003a, 2003b; Strack et al., 2003; von Schwedler et al., 2003). Specifically, CHMP6 binds to all ESCRT-II components, each CHMP4 isoform (A, B, and C) binds to AIP-1/ALIX, and CHMP5 binds to LIP5 (Ward et al., 2005). The remaining CHMP proteins CHMP1 (A and B), CHMP2 (A and B) and CHMP3 all bind to an ATPase VPS4 which plays an especially important role on the class E VPS pathway (see below). VPS4 may bind to additional, perhaps all, CHMP proteins although there is some disagreement in the literature on this point (Martin-Serrano et al., 2003a; von Schwedler et al., 2003). In general, while there are some differences among various studies in the details of precisely which CHMP proteins bind to each other and to VPS4, the anatomy of ESCRT-III appears to be largely conserved in yeast and humans (Babst et al., 2002a; Bowers et al., 2004; Martin-Serrano et al., 2003a, 2003b; von Schwedler et al., 2003).

There are a few reasons to think that CHMP proteins and VPS4 comprise the core machinery that mediates the membrane remodeling and/or fission events that separates nascent virions or vesicles from their parent membranes. First, catalytically inactive VPS4 mutants have a dominant negative (DN) phenotype and their expression induces the formation of class E compartment-like aberrant endosomes in both yeast and mammalian cells (Babst et al., 1998; Bishop and Woodman, 2000) on which most all of the known components of the class E VPS machinery accumulate. Importantly, this manipulation inhibits retroviral budding in a general way, i.e. irrespective of which particular type of L-domain is carried by the respective virus (Bishop and Woodman, 2000; Martin-Serrano et al., 2003b; Tanzi et al., 2003). This pan-specific ability to inhibit retrovirus budding is shared by dominant negative versions of CHMP proteins that can be created by fusion of a bulky or multimerizing protein at amino or carboxy termini of certain CHMP proteins (Martin-Serrano et al., 2003a, 2003b; Strack et al., 2003; von Schwedler et al., 2003). In contrast, it is possible to create rather specific inhibitors of PTAP, PPXY or YPDL type L-domains by expressing truncated forms of their respective cofactors (Demirov et al., 2002; Martin-Serrano et al., 2003a, 2003b). In addition, siRNA-mediated depletion of Tsg101 inhibits PTAP type L-domain activity, but has only minimal effects on YPDL or PPXY L-domains (Garrus et al., 2001; Martin-Serrano et al., 2003b; Tanzi et al., 2003) while AIP-1/ALIX depletion profoundly attenuates YPDL L-domain function but barely affects PTAP and PPXY L-domains (Martin-Serrano et al., 2003a). Overall, these types of experiments suggest that certain components are exploited in an L-domain-specific manner to access the class E VPS machinery, while others, specifically CHMP proteins and VPS4, appear to play a more general and central role in enveloped virus release.

## HECT ubiquitin ligases are required for PPXY-type L-domain activity

PTAP- and YPDL-type L-domains bind directly to class E VPS factors, but although PPXY L-domains are clearly

dependent on the class E VPS pathway (they are sensitive to inhibition by VPS4 and CHMP protein-based dominant inhibitors) (Garrus et al., 2001; Martin-Serrano et al., 2003a, 2003b), they apparently access it in a different way. PPXY is a consensus sequence for interaction with WW domains, a protein module that is present in multiple copies in HECT (Homologous to E6AP C-terminus) ubiquitin ligases. While yeast has a single HECT ubiquitin ligase (Rsp5) (Huibregtse et al., 1995), humans encode at least nine (Rotin et al., 2000). The most widely studied member of this family of proteins, Nedd4, is recruited by PPXY motifs in, for example, the cytoplasmic domains of the amiloride-sensitive epithelial Na+ channel (ENaC) and induces its down regulation (Staub et al., 1996, 2000). In addition to central WW domains, this group of proteins also encodes an amino-terminal C2 domain that directs membrane binding and localization and a carboxy-terminal HECT ubiquitin ligase catalytic domain, that can modify substrates by the addition of ubiquitin (Dunn and Hicke, 2001; Hicke, 2001).

Several studies have suggested that this family ubiquitin ligases might play a role in viral budding. For example, overexpression of various HECT ubiquitin ligase-derived WW domains can inhibit viral budding. In addition, the PPXY motifs present in the structural proteins of vesicular stomatitis virus (VSV) (Harty et al., 1999), Ebola virus (Harty et al., 2000; Yasuda et al., 2003), Rous sarcoma virus (RSV) (Kikonyogo et al., 2001), human T-cell leukemia virus (Blot et al., 2004; Bouamr et al., 2003; Heidecker et al., 2004; Sakurai et al., 2004) and Mason Pfizer monkey virus (Yasuda et al., 2002) have been reported bind to various HECT ubiquitin ligases including Nedd4, LDI-1, 2, BUL1, WWP1, WWP2 and Itch. In addition, an ENaC-derived PPXY-containing peptide sequence that is capable of binding multiple HECT ubiqutin ligases exhibits L-domain activity in the context of retroviral Gag proteins (Strack et al., 2000, 2002). Finally, overexpression of certain HECT ubiqutin ligases can modestly increase virus budding in some cases (Sakurai et al., 2004; Yasuda et al., 2002, 2003) and our own studies show that WWP1, WWP2 or Itch overexpression rescues a specific release defect that has been induced by overexpression of a WW domain protein fragment or by PPXY motif mutation (Martin-Serrano et al., 2005). Importantly, rescue of specific release defects required that the rescuing HECT ubiquitin ligase be catalytically active (Martin-Serrano et al., 2005).

Several HECT ubiquitin ligases are ubiquitously expressed (Wood et al., 1998) and could play redundant roles in PPXY-dependent viral budding. However, of the other HECT ubiquitin ligases tested, all bind to a more limited subset of viral PPXY motifs than do WWP1, WWP2 and Itch (Martin-Serrano et al., 2005). In addition, WWP1, WWP2 and Itch are unique among HECT ubiquitin ligases in that they can be convincingly demonstrated to be recruited to sites of viral budding by MLV Gag or Ebola virus matrix proteins, and fragments of them can be demonstrated to specifically induce a late budding defect in MLV (Martin-Serrano et al., 2005). Nonetheless, precisely which HECT ubiquitin ligase is used during viral budding probably depends on expression levels in infected tissues, perhaps on its subcellular localization, and the particular sequence context of the viral PPXY motif.

## How do ubiquitin and ubiquitin ligases mediate viral budding?

While recent studies demonstrate that the HECT active site, and presumably therefore, ubiquitination of some substrate protein, is required for PPXY-dependent viral budding, the functional target of ubiquitination during viral budding remains unclear. The most obvious candidates are the viral structural proteins themselves and several studies indicate that PPXY motifs induce ubiquitination of retroviral Gag proteins (Blot et al., 2004; Martin-Serrano et al., 2004; Ott et al., 2000; Strack et al., 2000). In addition, there is one example where mutation of multiple ubiquitin acceptor sites in RSV Gag has been shown to inhibit virus release (Spidel et al., 2004). In principle, deposition of ubiquitin, be it on virion or bystander proteins, may stabilize recruitment class E VPS factors, several of which bind ubiquitin (Alam et al., 2004; Bishop et al., 2002; Garrus et al., 2001; Polo et al., 2002), at sites of viral budding. Alternatively, cellular factors, including the class E VPS factor Hrs, may also be ubiquitinated by HECT ubiquitin ligases (Marchese et al., 2003; Polo et al., 2002). There is clear evidence that Rsp5-dependent trafficking in yeast can require ubiquitination of substrates other than the cargo protein (Dunn and Hicke, 2001). In principle, therefore, ubiquitination of factors other than viral proteins could influence their ability to promote viral budding.

While HECT ubiquitin ligases were thought to influence the fate of transmembrane proteins primarily because of their ability to modify transmembrane protein substrates or essential *trans*-acting factors, two lines of evidence suggest that HECT domains have additional functions. First, overexpressed WWP1 fragments that lack a HECT domain are markedly more potent inhibitors of PPXY-dependent viral budding than are those carrying catalytically inactive HECT domains (Martin-Serrano et al., 2005). This suggests that catalytically inactive HECT domains may retain some residual function. Second, the isolated HECT domain of WWP1, which does not ordinarily bind to membrane, appears to be a sufficient signal for recruitment to class E-like compartments induced by a expression of a catalytically inactive VPS4 mutant in mammalian cells (Martin-Serrano et al., 2005). While there are no known interactions between HECT domains and class E VPS factors, this finding suggests that some interaction between HECT domains and some component of the class E pathway exists, most likely involving an unidentified bridging factor.

A remaining question is whether ubiquitination in any form is required for release of viruses that encode PTAP or YPXL/LXXLF-type L-domains. Recent evidence suggests that the ability of viral L-domains to promote HIV-1 particle release correlates with their ability to reduce rather than increase the small amount of ubiquitin carried by the HIV-1 Gag protein (Gottwein and Krausslich, 2005; Martin-Serrano et al., 2004). Although mammalian class E VPS factors have not yet been unequivocally demonstrated to recruit deubiqui-

tinating enzymes, this clearly occurs in yeast (Amerik et al., 2000) and one mammalian class E VPS factor, namely HPB/STAM, has been reported to bind to the deubiquitinating enzyme, UBPY (Kato et al., 2000). Thus, correlating the extent of viral protein ubiquitination with the efficiency of virus release is difficult and likely confounded by several variables in particular the potentially numerous ubiquitin ligases and deubiquitinating enzymes that may be associated with the various components of the mammalian class E VPS machinery.

## Concluding perspectives and remaining questions

While recent years have seen rather dramatic advances in understanding of how enveloped viruses achieve separation from the cell that gave rise to them, several pressing questions remain. A central question is in the precise role of ubiquitin modification in virus release. This remains enigmatic, and while the above discussion communicates several plausible hypotheses, none has unequivocal experimental support. Treatment of cells with proteasome inhibitors can induce an L-domain mutant-like budding defect (Ott et al., 2003; Patnaik et al., 2000; Schubert et al., 2000) and it is presumed, but not clearly demonstrated, that this is due to depletion of free ubiquitin (Patnaik et al., 2000). Proteasome inhibition could have rather pleiotropic effects, perhaps including generalized inhibition of the class E VPS pathway. A vexing issue is that several of the candidate ubiquitin-binding or ubiquitin-modified proteins among the class E VPS factors appear not to be required in a general manner for viral budding (Alam et al., 2004; Bishop et al., 2002; Garrus et al., 2001; Polo et al., 2002). This may be due to redundancy, but nonetheless, this makes a demonstration of a functional role somewhat difficult. An additional potential problem in assigning specific roles to particular factors and L-domains in virus release is the possibility (or probability) that there remain more of each to be discovered. The known class E VPS proteins were largely discovered based on their ability to induce sorting defects in yeast when mutated (Katzmann et al., 2002; Raiborg et al., 2003) but this, by definition, required that the yeast remained viable. There may very well be additional complexity in the mammalian machinery and perhaps additional undiscovered factors are crucial for viral budding. Similarly, surveys of viral structural proteins for viral L-domain motifs are far from exhaustive. Most work on various L-domain motifs depends on a few seminal descriptions of PTAP, PPXY and YPDL motifs in retroviruses. The recent discovery of a completely novel L-domain in paramyxoviruses (Schmitt et al., 2005) illustrates that the L-domain 'database' is unlikely to be complete. Indeed, the recent appreciation that L-domains can exhibit quite strong context dependence (Martin-Serrano et al., 2004; Strack et al., 2002), could be explained by the presence of undiscovered underlying L-domains that act in a combinatorial manner to stimulate factor recruitment and virus release.

At present, we also do not have a clear idea as to what role each class E VPS protein plays in virus release and whether requirement for various activities differs among different viruses. The various morphologies of budding structures that are induced during virion formation might impose differing requirements for L-domain-recruited factors. In their cellular context, it appears that class E VPS factors should mediate at least three distinct activities, namely (i) the sorting of cargo proteins, (ii) membrane invagination to form a budding vesicular structure and (iii) membrane fission to form an intact vesicle. Different viruses might depend on these activities to differing extents, as such require recruitment of partial or complete subsets of class E VPS factors. Consistent with this idea, mutation of the PPXY motifs found in MPMV or HTLV-I a defect in which budding is arrested at an earlier stage than is typical of other retroviruses harboring mutant L-domains (Gottwein et al., 2003; Le Blanc et al., 2002). In addition, inhibition of MLV L-domain function can lead to the formation of tubes rather than the virtually complete spherical virions that characterize other retroviral L-domain mutants (Martin-Serrano et al., 2003a; Yuan et al., 2000) (Fig. 1B). Thus, L-domains could indeed recruit factors that play an important role in virion morphogenesis, dependent on the contribution of the viral structural proteins to defining particle morphology.

Nonetheless, it does seem that the minimal contribution that class E VPS factor recruitment makes is to induce fission of virion and cellular membranes. At present, it is unknown what constitutes the minimal components of the class E VPS pathway required for this step, but it seems most likely that the CHMP proteins and VPS4 are responsible, given their general requirement in viral budding. Activities such as the membrane curving activity ascribed to AIP-1/ALIX (Matsuo et al., 2004) do not appear to be required in a general sense for viral budding, because depletion of this protein using siRNA-based approaches does not have major impact on virus release that is mediated by PTAP or PPXY motifs (Martin-Serrano et al., 2003a). Among the CHMP proteins, work has centered around dominant negative fusion proteins and, surprisingly, even some CHMP proteins appear dispensable for the release of HIV-1 (Ward et al., 2005). Given this uncertainty in the minimal protein machinery required for the induction of membrane fission, even a rudimentary understanding of the mechanics by which enveloped virus release occurs appears some way off.

Finally, an important practical question is whether the class E VPS machinery can be manipulated in order to attenuate viral replication for therapeutic benefit. At first glance, this seems unlikely, given the crucial role that the sorting of membrane proteins at the limiting membrane of endosomes plays in the life of cells, and the fact that the known L-domains appear to act by mimicking recruitment events that are normally mediated by cellular proteins. Nonetheless, it may be possible for limited inhibition of L-domain:class E VPS factor interactions to have some therapeutic role in the context of a life threatening acute viral infection. Given the small size of the know viral L-domains, and the fact that the PTAP motif, for example, binds within a pocket in Tsg101 (Pornillos et al., 2002a, 2002b), such an approach to developing broad spectrum antiviral appears possible.

Even if there is no immediate therapeutic benefit to be derived from recent discoveries of the mechanism by which enveloped virus bud from cells, it is clear that the unanticipated complexities in the way that host cells and viral proteins interact during viral budding have informed the fields of virology and cell biology in ways that are only beginning to be appreciated.

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