

Retrovirus budding

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Abstract

The release of retrovirus particles from the infected cell is greatly stimulated by short motifs, known as “late” or “L” domains, present within the Gag precursor protein. Three distinct classes of L domains have been identified; these bear the core sequence: Pro-Thr/Ser-Ala-Pro [P(T/S)AP], Pro-Pro-x-Tyr (PPxY), or Tyr-Pro-x-Leu (YPxL). A number of recent studies have demonstrated that L domains function by interacting with components of the machinery responsible for sorting cellular proteins into the multivesicular body (MVB) pathway. This review traces the history of L domain discovery and characterization, and highlights the relationship between L domain activity, retrovirus release, and the host endosomal sorting machinery.

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Keywords: Retrovirus; L domains; Multivesicular

1. Introduction

Retroviral Gag proteins are synthesized in the cytoplasm of the infected cell and assemble into virus particles that typically bud from the plasma membrane. While the envelope glycoproteins and the *pol*-encoded enzymes are required for the production of infectious progeny virions, expression of Gag proteins alone is generally sufficient for the assembly and release of non-infectious, virus-like particles (VLPs). Retrovirus assembly itself usually takes place at either of two subcellular locations (Goff, 2001): for type C retroviruses, which include the alpharetroviruses, gammaretroviruses, and lentiviruses, the assembly of electron-dense structures occurs at the plasma membrane. For type B and D retroviruses, assembly takes place in the cytosol and the assembled intracytoplasmic particles (known as A-type particles) traffic to the plasma membrane where they bud from the cell. The mature Gag proteins [matrix (MA), capsid (CA), and nucleocapsid (NC)] are generated concomitant with virus release upon cleavage of the Gag precursor by the viral protease (PR)

(Fig. 1). PR-mediated Gag processing leads to virus maturation, a morphological transition essential for virus infectivity. While maturation most commonly occurs at the plasma membrane, this PR-induced phenomenon can also take place upon budding of particles into the lumen of intracellular structures known as multivesicular bodies (MVBs). In addition to MA, CA, and NC, the *gag* genes of individual retrovirus genera often encode additional domains and spacer peptides (Fig. 1). Discrete regions have been identified within retroviral Gag precursor proteins that orchestrate the major steps in virus assembly and release: the membrane binding (M) domain directs the association of Gag with membrane, typically through a bipartite motif consisting of a covalently attached myristic acid moiety and a highly basic domain in MA. The interaction (I) domain promotes Gag–Gag multimerization that drives the assembly process, and the L domain functions late in the assembly pathway to stimulate the release of virus particles from the plasma membrane. This chapter will focus on the role of L domains in retrovirus release and on the interplay between L domains and host factors. Because of their widespread importance in retrovirus budding, the associations between L domains and components of the cellular endosomal sorting machinery will be emphasized.

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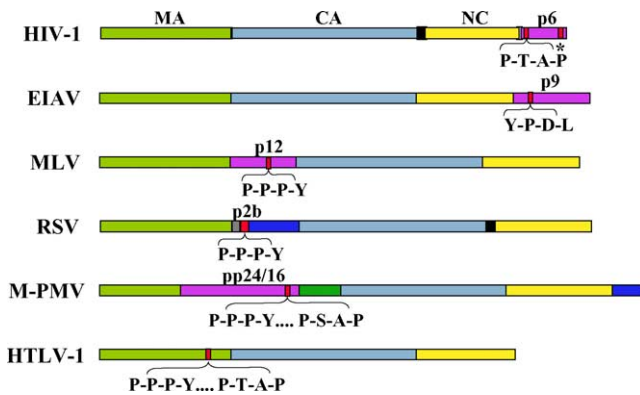


Fig. 1. Schematic representation of retroviral Gag proteins and the location of retroviral L domains. Matrix (MA), capsid (CA), and nucleocapsid (NC) domains are shown in light green, light blue and yellow, respectively. Gag proteins that harbor L domains are depicted in purple with the exception of HTLV-1, where the L domain is located in MA. The L domains are marked in red. The asterisk indicates the location of the AIP1/Alix-binding sequence (LYPLASL) in p6. Nucleotide accession numbers of the molecular clones listed in the figure are given below: HIV-1—M19921 (NL4-3); AF033819 (HXB2CG). EIAV—M16575 (EIAVCG). MLV—J02255 (MLMCG). RSV—V01197 (Prague C). M-PMV—M12349 (MPMV/6A). HTLV-1—AF033817. An extensive list of retroviral genomes can be found at: (<http://www.ncbi.nlm.nih.gov/retroviruses/>).

2. L domains harbor protein binding motifs

2.1. L domain consensus sequences: P(T/S)AP, PPxY, and YPxL

The first suggestion that retroviruses harbor regions that are necessary for virus release arose from the observation that truncation of the p6 domain at the C-terminus of HIV-1 Gag (Fig. 1) markedly inhibited virus production at a late stage in the assembly/release process (Gottlinger et al., 1991). The truncated Gag protein assembled into virus-like structures at the plasma membrane, but rather than being released into the extracellular space the mutant particles remained connected to the cell surface by a thin tether or stalk (Fig. 2A). Extensive mutational analysis of HIV-1 p6 revealed that a highly conserved stretch of amino acids with the consensus sequence Pro-Thr/Ser-Ala-Pro [hereafter referred to as P(T/S)AP] (Fig. 1) was responsible for the budding and release activity of p6 (Huang et al., 1995). Even subtle mutations in this motif caused a severe defect in virus particle production (Demirov et al., 2002b; Huang et al., 1995). L domains with P(T/S)AP motifs were subsequently identified in simian immunodeficiency virus (SIV), HIV-2, Mason-Pfizer monkey virus (M-PMV), and human T-cell leukemia virus (HTLV-1) (Fig. 1) (Bouamr et al., 2003; Gottwein et al., 2003; Myers and Allen, 2002; Wang et al., 2004). P(T/S)AP-type L domains are also found outside the retrovirus family; e.g., in Ebola virus (see chapter by Jasenosky and Kawaoka). It initially appeared likely that the P(T/S)AP-type L domain motif was a member of the class of Src homology 3 (SH3)-binding modules that possess PxxP core interacting signals (Mayer,

2001), in particular, since one class of SH3-binding domain contains the consensus sequence (R/K)xxPxxP that is found in p6. However, mutation of the consensus Arg residue in this sequence does not inhibit virus budding and the residues between the prolines, which are relatively unimportant in the context of SH3-binding motifs, are critical to p6 L domain function (Demirov et al., 2002b; Huang et al., 1995). These results suggested that P(T/S)AP L domain motifs were perhaps not SH3 interacting sequences (Demirov et al., 2002b), and, indeed, this motif was found to recognize a novel protein-binding domain (Pomillos et al., 2002a, b) (see below).

Mutational analysis of the Gag protein of the avian alpharetrovirus Rous sarcoma virus (RSV) identified a Pro-rich motif, specifically Pro-Pro-Pro-Tyr (PPPY), that is necessary for efficient virus particle release from Gag-expressing cells (Wills et al., 1994; Xiang et al., 1996). This L domain motif is located in a small spacer peptide, known as p2b, situated between the MA and CA domains of RSV Gag (Fig. 1). Subsequent to the identification of the RSV PPPY sequence as an L domain, analogous motifs with the PPxY consensus sequence were defined in several other retroviruses including M-PMV (Yasuda and Hunter, 1998), murine leukemia virus (MLV) (Yuan et al., 1999), HTLV-1 (Heidecker et al., 2004; Le Blanc et al., 2002b; Wang et al., 2004) and bovine leukemia virus (BLV) (Wang et al., 2002) (Fig. 1). Mutations in PPxY motifs often result in virion-cell tethering defects similar to those observed with HIV-1 L domain substitutions (Fig. 2A). In some cases, however, an earlier block is observed, visualized by EM as the accumulation of electron dense patches at the plasma membrane (Fig. 2A). These results suggest that PPxY motifs may function not only in virion release but also in bud formation.

The presence of an L domain in the p9 portion of the Gag protein of the non-primate lentivirus equine infectious anemia virus (EIAV) (Fig. 1) was revealed by the observation that substitution of p9 for RSV p2b rescued the virus release defect imposed by p2b deletion (Parent et al., 1995). Mutational analysis of EIAV p9 mapped this L domain activity to a Tyr-Pro-Asp-Leu (Y-P-D-L) sequence (Parent et al., 1995; Puffer et al., 1997). This core motif conforms to the YPxL/I protein-binding determinant (Vincent et al., 2003). A related sequence (YPLASL) is found near the C-terminus of HIV-1 p6 and, in certain contexts, this motif can provide some L domain activity to HIV-1 (Martin-Serrano and Bieniasz, 2003; Strack et al., 2003). Interestingly, a similar sequence, LYPSL, is located in the p6 domain of an SIV isolate, SIV_{col}, that lacks a P(T/S)AP motif. These observations suggest that either SIV_{col} harbors a novel L domain or that, for a limited number of lentiviruses or in particular cell types, this p6 sequence might function as a primary L domain (Courgnaud et al., 2001; Strack et al., 2003).

Several retroviruses, including M-PMV and HTLV-I, contain both P(T/S)AP and PPPY L domains (Fig. 1) (Blot et al., 2004; Bouamr et al., 2003; Gottwein et al., 2003; Heidecker et al., 2004; Sakurai et al., 2004; Wang et al., 2004). When both sequences are present the PPPY motif appears to predominate

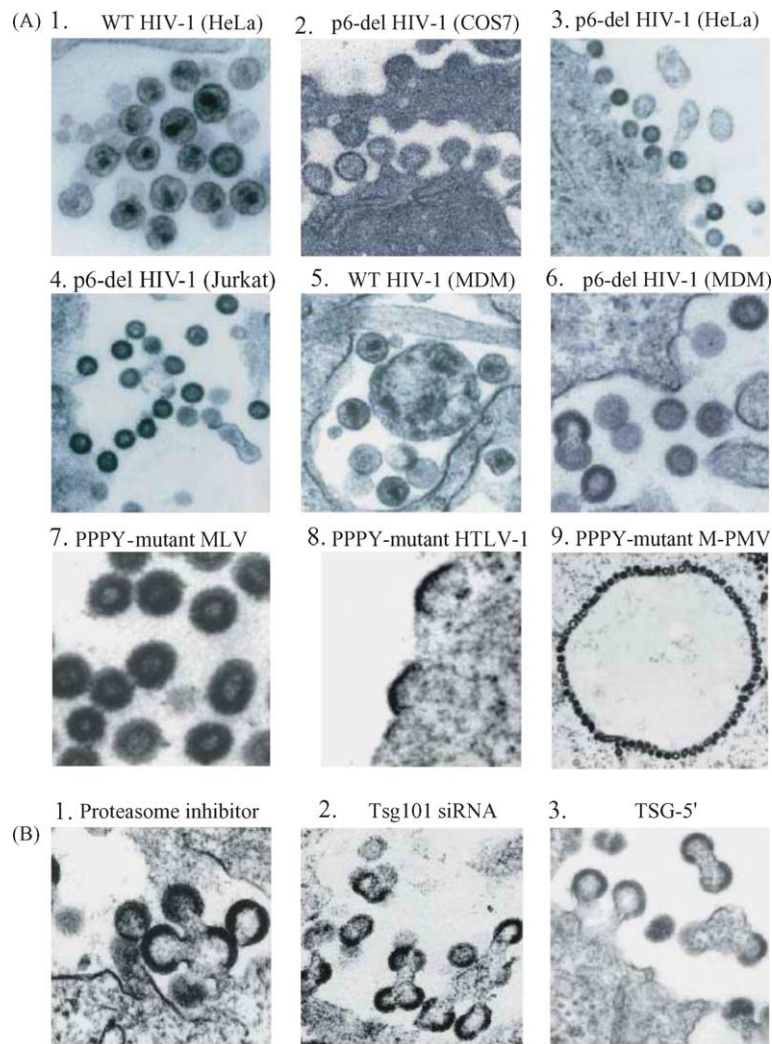


Fig. 2. EM visualization of virus budding defects induced by retroviral L domain mutations (A) or by HIV-1 release inhibitors (B) p6-del, p6-deleted; MDM, monocyte-derived macrophages. Refs: *Panel A1, A3, A4, A5, A6* (Demirov et al., 2002b); *A2* (Gottlinger et al., 1991); *A7* (Yuan et al., 1999); *A8* (Le Blanc et al., 2002b); *A9* (Gottwein et al., 2003); *B1* (Schubert et al., 2000); *B2* (Garrus et al., 2001); *B3* (Demirov et al., 2002a). For panels A1 and A3–A9, copyright American Society for Microbiology; A2, B1, and B3, copyright National Academy of Sciences, U.S.A.; B2, copyright 2001, Elsevier.

inate. Interestingly, mutations in the M-PMV and HTLV-1 P(T/S)AP motif generally give rise to a late budding defect, with particles tethered to the plasma membrane. In contrast, mutations in PPPY appear to result in an earlier defect, manifested by the accumulation of electron-dense patches of Gag at the plasma membrane (Gottwein et al., 2003; Le Blanc et al., 2002b). (Fig. 2A). Consistent with the dominant role of PPPY in HTLV-I release, the P(T/S)AP motif can be functionally replaced by PPPY or YPDL whereas the PPPY motif cannot be substituted (Wang et al., 2004). These results suggest that at least for M-PMV and HTLV-1, P(T/S)AP and PPPY L domains are not simply redundant but may perform distinct roles in virus budding and release.

L domains harbor Ser/Thr or Tyr residues that may be substrates for phosphorylation, and many interactions among cellular proteins that harbor sequences related to those found in retroviral L domains are regulated by phosphorylation. It is therefore reasonable to speculate that L domain phosphoryla-

tion may play a role in virus budding. This mechanism of regulation could affect interactions between L domains and their host partners in either a positive or negative manner (Neel et al., 2003; Ravetch and Lanier, 2000; Sudol and Hunter, 2000). Several observations are consistent with the possibility that L domain function could be regulated by phosphorylation: (i) The L domain-containing Gag proteins of several retroviruses (e.g., HIV-1 p6, MLV p12, and M-PMV pp24/16) are phosphorylated (Bradac and Hunter, 1984; Muller et al., 2002; Sen et al., 1977), and, at least in the case of HIV-1 p6, phosphorylation appears to be a late event (Muller et al., 2002). (ii) Endocytosis and sorting of several plasma membrane proteins is sequentially regulated by phosphorylation and ubiquitylation (Hicke, 1999). (iii) The ERK2 kinase, whose consensus target sequence is closely related to P(T/S)AP, is incorporated into HIV-1 virions (Cartier et al., 1997). Finally, (iv) the pinching-off of virions from the plasma membrane is inhibited by ATP-depleting agents (Tritel and Resh, 2001). Despite the

importance of phosphorylation in regulating a number of cellular protein–protein interactions, a direct link between phosphorylation and retrovirus release remains to be demonstrated.

2.2. L domains often display positional independence and functional exchangeability

The mutational analyses discussed above indicate that retroviral L domains are highly conserved and are relatively short, linear motifs analogous to protein-binding modules previously characterized in cellular proteins. These observations led to the suggestion that L domains serve as docking sites for host factors. This hypothesis was further supported by a number of reports demonstrating that L domains can be exchanged between viruses and can function at various positions within Gag. For example, as mentioned above, EIAV p9 can replace the L domain activity of RSV p2b (Parent et al., 1995). In addition, the Ebola virus L domain (which is composed of overlapping PTAP and PPEY sequences) restores the release of p2b-deficient RSV Gag (Craven et al., 1999). In turn, RSV p2b can promote budding of L domain-deficient MLV and EIAV particles (Li et al., 2002; Yuan et al., 2000), and a region of p6 spanning the P(T/S)AP motif replaces the requirement for a PPPY motif in MLV particle budding and infectivity (Yuan et al., 2000). Budding of a highly truncated “minimal” assembly-competent HIV-1 Gag can be restored by the L domains of RSV (Accola et al., 2000), EIAV (Strack et al., 2003), and Ebola virus (Strack et al., 2002). Interestingly, the p6-derived P(T/S)AP core motif is not sufficient to provide L domain activity when placed at the C-terminus of the minimal HIV-1 Gag (Strack et al., 2002). In addition, although heterologous L domains can function when fused to minimal HIV-1 Gag constructs, they do not function efficiently in the context of bona fide, full-length HIV-1 Gag (Shehu-Xhilaga et al., 2004). Finally, PPxY-type L domains appear to be non-functional in the HIV-1 context (Martin-Serrano et al., 2004). Collectively, these results indicate that L domains are often exchangeable, but in certain circumstances display context dependence.

2.3. L domains function in a cell-type-dependent manner

While the major defect caused by L domain mutation is a failure of particles to pinch off from the plasma membrane, under certain conditions this phenotype is not observed. For example, the release of HIV-1 VLPs from baculovirus-infected insect cells (Jowett et al., 1992; Royer et al., 1991) or from vaccinia virus-infected mammalian cells (Hoshikawa et al., 1991; Spearman et al., 1994) is apparently insensitive to L domain deletions. A plausible explanation for the lack of a defect in particle production is that high Gag expression levels may obscure L domain requirements and/or may reveal the importance of additional regions in Gag that harbor redundant, less efficient L domains. It has also been observed that release defects induced by L domain mutations are, in

some systems, less severe in the absence of a functional PR (Huang et al., 1995; Wang et al., 2002).

A lack of virus release defect was also observed in T cells expressing p6 L-domain-mutant HIV-1 (Demirov et al., 2002b; Schwartz et al., 1996). However, EM analysis demonstrated that in T cells L-domain-mutant virions are released from the cell surface but fail to detach from each other, producing chains of virus particles (Fig. 2A, panel 4) (Demirov et al., 2002b). Particles tethered to each other or to the cell surface display a predominantly immature morphology (Demirov et al., 2002b). Virion–virion tethering has also been observed for MLV L domain mutants (Yuan et al., 2000) (Fig. 2A, panel 7). While it remains to be defined why L domain mutants display predominantly virion–cell tethering in some cell types and virion–virion tethering in others, these observations indicate that the requirements for virus release are, to some extent, cell-type-dependent.

3. The role of ubiquitin in virus release

Ubiquitylation is a post-translational protein modification that serves to tag target proteins for particular cellular responses. Ubiquitin (Ub) is a small, highly abundant protein that can be covalently attached to certain Lys residues in target proteins (Hershko and Ciechanover, 1998). Ubiquitylation takes place in a series of steps requiring the sequential action of three enzymes: (i) an activating enzyme (E1) forms a thiol ester linkage between one of its Cys residues and the C-terminal Gly of Ub; (ii) the activated Ub is transferred to a conjugating enzyme (E2); and, (iii) the conjugated Ub moiety is transferred to a Lys residue on the target protein, usually through the action of a Ub ligase (E3). Ub can also be covalently linked to other Ub moieties on target proteins to form polyubiquitin chains. Mammalian cells are thought to contain a single E1, dozens of E2s, and perhaps hundreds of E3s (Hershko and Ciechanover, 1998). In addition to this great diversity of E2s and E3s, a family of E2-related proteins performs less well-characterized functions in the cell. These proteins, referred to as Ub E2 variants (UEVs), lack the active-site Cys required for covalent linkage to Ub but nevertheless retain the ability to bind Ub. An example of a protein that carries a UEV domain is Tsg101 (Koonin and Abagyan, 1997), which will be discussed in more detail below. Many aspects of ubiquitylation remain incompletely characterized; for example, what determines the substrate specificity for particular target proteins, how are specific Lys residues selected for modification, and what dictates whether a protein will be mono- or poly-ubiquitylated (Haglund et al., 2003). It is clear, however, that poly-Ub chains (containing more than four Ub moieties) generally serve as a signal for protein degradation in the proteasome, whereas monoubiquitylation often tags membrane-associated proteins for internalization and/or sorting into the endosomal pathway.

Multiple lines of evidence suggest a connection between Ub and retrovirus release (Vogt, 2000): (i) several retroviruses

contain free, virion-associated Ub (Putterman et al., 1990) and a small amount of monoubiquitylated Gag (Heidecker et al., 2004; Ott et al., 1998, 2002). (ii) Proteasome inhibitors, which deplete free Ub by inducing the accumulation of polyubiquitin complexes, disrupt the release of some (but not all) retroviruses (Accola et al., 2000; Ott et al., 2002, 2003; Patnaik et al., 2000; Schubert et al., 2000). (iii) The defect in RSV release imposed by proteasome inhibitors can be partially rescued by fusing a Ub moiety to the C-terminus of RSV Gag (Patnaik et al., 2000). (iv) Overexpression of a mutant form of Ub lacking residues implicated in Ub-regulated endocytosis inhibits HIV-1 release (Strack et al., 2002). Finally, (v) retroviral L domains interact directly with components of the ubiquitylation machinery (see below).

While it is well accepted that proteasome inhibitors disrupt the release of a number of retroviruses, the basis for this inhibition remains to be defined. It has been postulated that by depleting intracellular pools of free Ub, proteasome inhibitors limit the extent to which Gag is ubiquitylated, thereby directly disrupting virus budding. The hypothesis that Ub plays a direct role in virus release is supported by the above-mentioned finding that the production of VLPs generated with an RSV Gag-Ub fusion protein is partially resistant to proteasome inhibitors (Patnaik et al., 2000). It is also clear, as will be discussed in more detail below, that Ub often plays a key role in the recognition of cargo proteins by the endosomal sorting machinery (Hicke and Dunn, 2003). Several observations, however, raise the possibility that the attachment of Ub to Gag may be an indirect consequence of L domain-mediated recruitment of ubiquitylation machinery to the site of budding. (i) Treatment of cells with proteasome inhibitors affects the trafficking of multiple cellular proteins and may induce the type of aberrant endosomal sorting defect that is known to block virus release (Longva et al., 2002; van Kerkhof et al., 2001; van Kerkhof et al., 2000). (ii) Substitution or deletion of Lys residues that are targets for ubiquitylation in HIV-1 p6 or MLV p12 has no effect on virus release or replication (Demirov et al., 2002b; Ott et al., 2000), though it has not been determined whether alternative Lys residues are ubiquitylated in these mutants. (iii) Brief treatment of virus-producing cells with proteasome inhibitors impairs virus production, despite the fact that these treatments do not significantly deplete free Ub (Ott et al., 2003). (iv) MMTV and EIAV Gag proteins are ubiquitylated, yet the release of these viruses is insensitive to proteasome inhibitors (Ott et al., 2002; Patnaik et al., 2002). Sequence analysis of EIAV p9 suggested the presence of a short motif with some homology to Ub. This observation led to the hypothesis that the Ub-related sequence in p9 renders ubiquitylation of EIAV p9 unnecessary, thus providing an explanation for the resistance of EIAV release to proteasome inhibitors (Patnaik et al., 2002). However, a recent study demonstrated that the release of EIAV p9 mutants in which the native YPDL L domain was exchanged for PPPY or PTAP was sensitive to proteasome inhibitors, suggesting that resistance or sensitivity to these compounds is determined by the L domain itself and not by sequences in Gag outside the L

domain (Shehu-Xhilaga et al., 2004). Together, these results indicate that the functional relevance to particle budding of Gag modification by Ub remains to be established. It is conceivable that the importance of Gag ubiquitylation could differ depending on which L domain class(es) are utilized by a particular retrovirus, and could even vary with other factors such as cell-type and expression conditions.

4. L domains interact with components of the cellular endosomal sorting machinery

A key step in the trafficking of membrane proteins and lysosomal enzymes to the lysosome (or the vacuole in yeast) is the delivery of these proteins to the late endosome prior to their fusion with the lysosome. In many cases, lysosomally targeted proteins are marked for sorting by monoubiquitin tags. The cargo proteins are initially delivered to early endosomal membranes where they are incorporated into vesicles (intraluminal vesicles or ILV) that bud into the lumen of the organelle. Early endosomes mature to late endosomes, or MVBs, that accumulate multiple ILVs (Jiang et al., 2002; Katzmann et al., 2002; Piper and Luzio, 2001; Raiborg et al., 2003). Importantly, MVB biogenesis is topologically equivalent to the budding of retrovirus particles; in both cases budding is directed away from the cytoplasm. Compelling data have been obtained from a number of labs indicating that retroviruses have evolved to hijack MVB machinery for their release from the cell.

4.1. The class E Vps machinery

Morphological characterization of vacuolar protein sorting (Vps) mutants in yeast led to the classification of a number of distinct vacuolar morphologies (Raymond et al., 1992). One morphological class ("class E") was defined by the formation of an exaggerated, pre-vacuolar compartment (the so-called "class E" compartment) in which Vps proteins accumulated (Katzmann et al., 2002). Class E Vps family members are cytoplasmic, multidomain proteins that transiently attach to the endosomal membrane where the inward invagination of cargo-laden vesicles takes place. Many class E Vps proteins assemble into discrete complexes termed ESCRT-I, -II, or -III (for endosomal sorting complex required for transport) (Babst et al., 2002a,b; Katzmann et al., 2001). The function of class E Vps proteins, and the multiprotein complexes into which they assemble, appears to be relatively well conserved from yeast to mammalian cells (Bowers et al., 2004; Katzmann et al., 2002; Martin-Serrano et al., 2003a; von Schwedler et al., 2003).

Cell surface expression of particular plasma membrane-associated receptors is regulated, upon ligand binding, by their internalization into early endosomes. Following endocytosis, the receptors are recycled back to the cell surface or sorted for degradation in the lysosome. Modification by monoubiquitylation often tags cargo proteins for

lysosomal targeting and degradation. In the early endosome, membrane-bound cargo associates with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), the mammalian cell homolog of the yeast class E Vps protein Vps27 (Piper and Luzio, 2001; Raiborg et al., 2003). The interaction between Hrs and cargo is governed by the recognition of the Ub tag by the Ub interacting motif (UIM) of Hrs (Hofmann and Falquet, 2001; Polo et al., 2002; Raiborg and Stenmark, 2002; Shih et al., 2002). Hrs in turn recruits ESCRT-I to the endosomal membrane by interaction with Tsg101 (Vps23 in yeast) (Bache et al., 2003; Katzmann et al., 2003; Pornillos et al., 2003). The interaction between Hrs and Tsg101 is mediated by two separate domains in both proteins: a PSAP motif in Hrs binds a P(T/S)AP-binding pocket in the UEV domain of Tsg101 and a PTAP motif in Tsg101 associates with a putative PT(S)AP-binding site within a Pro/Gln-rich domain of Hrs (Figs. 3 and 4) (Amara and Littman, 2003; Bache et al., 2003; Katzmann et al., 2003; Lu et al., 2003; Pornillos et al., 2003). The cargo-bound ESCRT-I complex then recruits ESCRT-II and ESCRT-III to the membrane. Yeast ESCRT-II consists of Vps22, 25, and 36 and the ESCRT-III complex is composed of two subcomplexes: Vps2/Vps24 and Snf7/Vps20 (Babst et al., 2002a,b). The mammalian cell homologs of the ESCRT-II proteins are Eap25, Eap30, and Eap45; and the so-called CHMP proteins (for chromatin modifying protein or charged multivesicular body protein) (Stauffer et al., 2001) constitute the ESCRT-III homologs (Katzmann et al., 2002). The disassembly of the ESCRT complexes at the site of membrane invagination is promoted by the enzymatic activity of the AAA (for ATPase associated with various cellular activities) ATPase Vps4 (Babst et al., 2002a; Bishop and Woodman, 2000; Yeo et al., 2003). Overexpression of ATPase-deficient Vps4 mutants in mammalian cells induces the formation of swollen, aberrant endosomes analogous to the class E compartment that results from inactivation of class E Vps proteins in yeast (Bishop and Woodman, 2000). Subsequent fusion of the MVB with lysosomes (or the vacuole in yeast) leads to the degrada-

tion of cargo proteins through the action of lipases and proteases.

Recent studies have shed light on another notable player in the MVB pathway, known variously as Bro I or Vps31 in yeast and AIP1 (ALG2-interacting protein 1) or Alix (ALG2-interacting protein X) in mammalian cells (Figs. 3 and 4). In yeast, BroI/Vps31 was recognized for its involvement in the Ub-dependent downregulation of the general amino acid permease, Gap1 (Springael et al., 2002). As with other class E Vps proteins, BroI/Vps31 accumulates in the class E compartment in *vps4*-deficient yeast (Odorizzi et al., 2003). While not part of any of the three recognized ESCRT complexes, this protein associates with ESCRT-III components in both yeast and mammalian cells (Martin-Serrano et al., 2003a; Odorizzi et al., 2003; von Schwedler et al., 2003). In mammalian cells, AIP1/Alix appears to serve as a bridge between ESCRT-I and ESCRT-III complexes by simultaneously interacting with Tsg101 and CHMP proteins (Fig. 4) (Martin-Serrano et al., 2003a; von Schwedler et al., 2003). AIP1/Alix also reportedly regulates the interaction of intraluminal vesicles with the limiting membrane of lysobisphosphatidic acid (LBPA)-containing endosomal membranes (Dikic, 2004; Matsuo et al., 2004). AIP1/Alix has recently attracted the attention of retrovirologists as the cellular interacting partner for the EIAV YPxL L domain and the putative secondary L domain of p6 (see below). Interestingly, in addition to its associations with ESCRT-I and -III, AIP1/Alix also binds endophilins (Chatellard-Causse et al., 2002), which possess lysophosphatidic acid acyltransferase activity implicated in the invagination and fission of endocytic vesicles at the plasma membrane (Reutens and Begley, 2002; Schmidt et al., 1999). Whether the endophilin-associating or LBPA-regulating activities of AIP1/Alix plays a role in retrovirus release remains to be determined. A role for endophilins in retrovirus particle production gains support from the observation that MLV MA interacts with endophilin 2, and overexpression of endophilin 2 fragments inhibits MLV release (Wang et al., 2003).

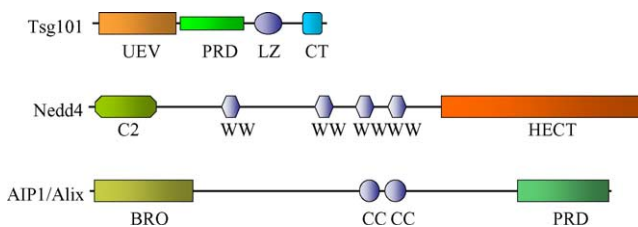


Fig. 3. Schematic representation of the cellular proteins that interact with L domains. Tsg101: UEV, ubiquitin E2 variant (or E2-like) domain; PRD, proline-rich domain; LZ, leucine zipper; CT, C-terminal domain; PTAP-type L domains interact with the UEV domain. Nedd4: C2, Ca²⁺-dependent membrane-binding domain; WW, WW domains; HECT (for homology to E6 C-terminus), catalytic E3 ligase domain; PPxY-type L domains interact with the WW domains of Nedd4 or related proteins. AIP1/Alix: BRO, a conserved motif with unknown function; CC, coiled-coil motifs; PRD, proline-rich domain; YPxL (or related) L domains interact with the PRD. Additional details provided in the text.

4.2. P(T/S)AP L domains interact with the ESCRT-I component Tsg101

A major advance in our understanding of retrovirus budding was provided by the finding that, in yeast two-hybrid screens, p6 interacts with the ESCRT-I component Tsg101, and that mutations in the p6 P(T/S)AP motif abrogates this interaction (Garrus et al., 2001; VerPlank et al., 2001). Binding between the p6 L domain and Tsg101 is also observed in mammalian cells and Tsg101 is found to be incorporated into virions in a P(T/S)AP-dependent manner (Demirov et al., 2002a; Goila-Gaur et al., 2003). The functional relevance of the p6-Tsg101 interaction is supported by several complementary findings: (i) depletion of endogenous Tsg101 using an siRNA approach inhibits virus release (Garrus et al., 2001), and (ii) overexpression of the N-terminal UEV domain of Tsg101 (Fig. 3) (referred to as TSG-5') disrupts HIV-1 bud-

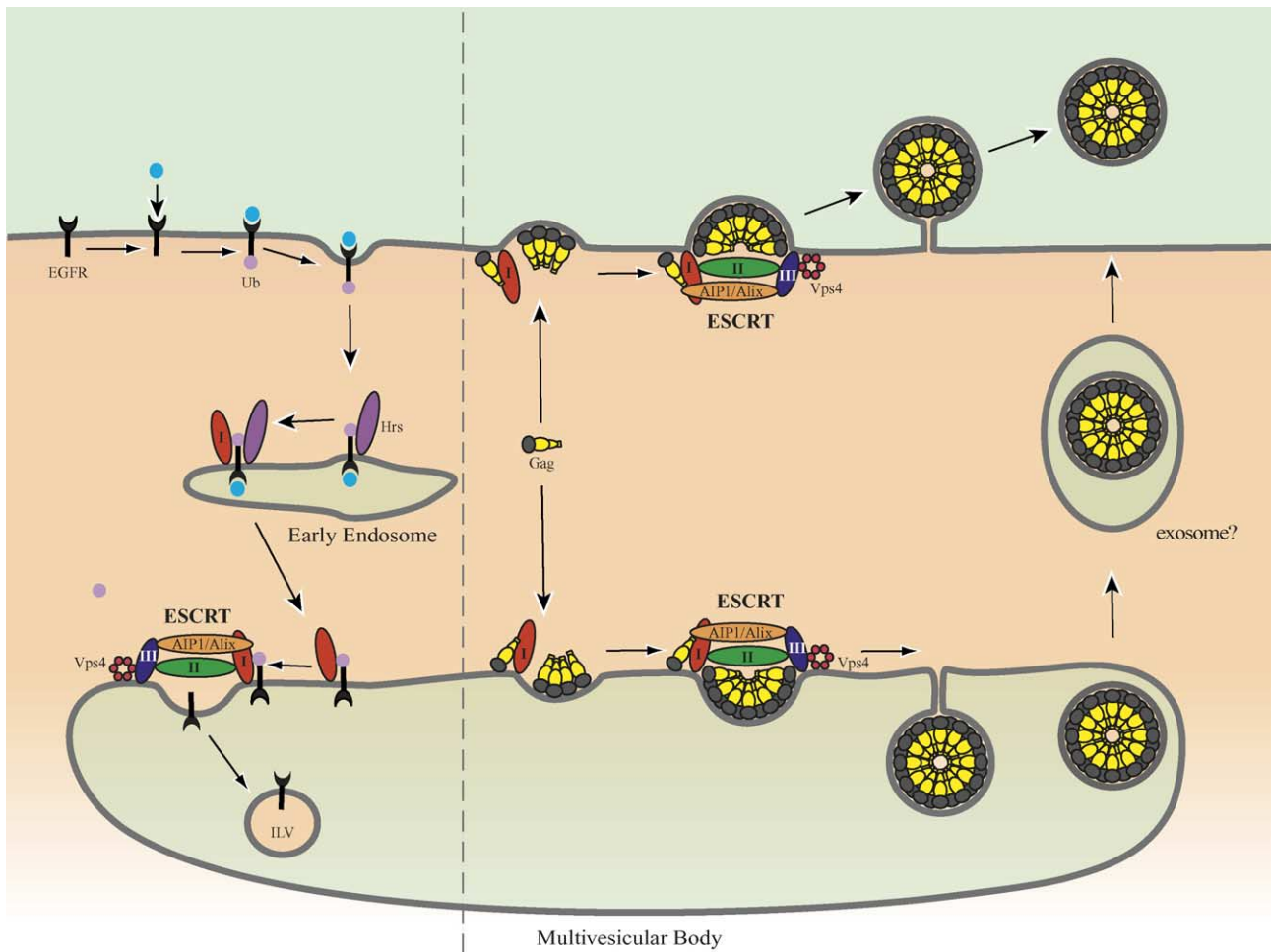


Fig. 4. Model for retrovirus release. On the left is a schematic representation of endocytosis and MVB sorting of an activated growth factor receptor. An Hrs-containing complex (dark purple) recognizes and sequesters ubiquitylated cargo (Ub, light purple) at clathrin-rich regions of the early endosomal membrane. Hrs recruits ESCRT-I (I, red) through a direct interaction with Tsg101. ESCRT-I in turn recruits additional components of the MVB pathway, i.e., ESCRT-II (II, green) and ESCRT-III (III, blue), to assemble into a functional sorting complex. AIP1/Alix (brown) is recruited to the assembled complex and in mammalian cells bridges ESCRT-I and -III. The AAA ATPase Vps4 (red) is critical for disassembly of the complex following inward budding of vesicles into the endosomal lumen. On the right is depicted the hijacking of MVB sorting machinery for virus release. HIV-1 Gag is believed to mimic Hrs in triggering membrane recruitment of ESCRT-I. Virus particles are shown to assemble and bud at the plasma or to be released (e.g., from macrophages) through the exosome pathway following assembly in the MVB. Additional details provided in the text.

ding (Demirov et al., 2002a) (Table 1). Both Tsg101 siRNA and TSG-5' produce a virion tethering defect essentially indistinguishable by EM from the defect induced by HIV-1 L domain mutations (Demirov et al., 2002a; Garrus et al., 2001) (Fig. 2B). In addition, a Gag-Tsg101 fusion protein is able to provide L domain function when co-expressed with an L-domain-mutant Gag (Martin-Serrano et al., 2001).

Tsg101 interacts with the p6 P(T/S)AP motif via its N-terminal UEV domain (Demirov et al., 2002a; Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001) (Fig. 3). NMR and X-ray crystallography studies of the Tsg101 UEV domain (Pornillos et al., 2002a,b; Sundquist et al., 2004), together with mutational analyses (Garrus et al., 2001; Goila-Gaur et al., 2003; VerPlank et al., 2001), revealed that the UEV domain harbors distinct binding sites

for P(T/S)AP and for Ub. The physiological role for the Ub and P(T/S)AP binding sites appears to be interaction with cargo-bound Ub and the PSAP motif of Hrs, respectively. Indeed, it has been proposed that HIV-1 Gag has evolved to mimic Hrs in its binding to Tsg101 (Pornillos et al., 2003). p6 ubiquitylation may enhance, but is not required for, the p6-Tsg101 association (Garrus et al., 2001; VerPlank et al., 2001). Overexpression of the Tsg101 UEV domain (TSG-5') inhibits HIV-1 release by interfering with the p6 L domain, as mutation of the P(T/S)AP binding site largely abrogates the ability of TSG-5' to inhibit virus release (Goila-Gaur et al., 2003). In addition, retroviruses (e.g. MLV and EIAV) that depend on non-P(T/S)AP L domains (e.g., PPxY or YPDL) for their release are not inhibited by TSG-5' (Demirov et al., 2002a; Shehu-Xhilaga et al., 2004). Further support for the

Table 1
Inhibitors of retrovirus release

Agent	Virus(es) inhibited	Proposed mechanism	Reference
Tsg101 siRNA	HIV-1, HTLV-I	Tsg101 depletion – disruption of ESCRT-I complex	Blot et al., 2004; Garrus et al., 2001; Martin-Serrano et al., 2003a,b
AIP1/Alix siRNA	Retroviral construct that harbors YPxL-type L domain	AIP1/Alix depletion	Martin-Serrano et al., 2003a,b
Proteasome inhibitors	HIV-1, SIV, MLV, RSV HTLV-I	ND ^a	Bouamr et al., 2003; Ott et al., 2003; Patnaik et al., 2000; Schubert et al., 2000; Strack et al., 2000
ATP-depleting agents	HIV-1	ND ^a	Tritel and Resh, 2001
Tsg101 N-terminal half (TSG-5')	HIV-1, HTLV-1, retroviral constructs that harbor PTAP-type L domain	Prevents interaction of PTAP-type L domain with the ESCRT machinery	Bouamr et al., 2003; Demirov et al., 2002a; Goila-Gaur et al., 2003; Shehu-Xhilaga et al., 2004
AIP1/Alix C-terminal half (AIP1ΔBRO, AIP1/Alix δ1-176)	HIV-1, EIAV	Prevents interaction of YPxL-type L domain with the ESCRT machinery	Martin-Serrano et al., 2003a,b; Strack et al., 2003
Fragments from Nedd4-related proteins (e.g., Nedd4, LDI-1, BUL1, WWP1)	RSV, M-PMV, HTLV-1	Prevents interaction of PPxY-type L domain with Nedd4-related proteins	Blot et al., 2004; Bouamr et al., 2003; Heidecker et al., 2004; Kikonyogo et al., 2001; Yasuda et al., 2002
Tsg101 C-terminal half (TSG-3')	HIV-1, MLV, retroviral constructs that harbor PTAP- or PPxY-type L domains	Disruption of class E Vps machinery	Goila-Gaur et al., 2003; Shehu-Xhilaga et al., 2004
AIP1/Alix N-terminal half (AIP1ΔPRD)	EIAV	ND ^a	Strack et al., 2003
Dominant negative Vps4A and 4B mutants (Vps4EQ)	HIV-1, MLV, EIAV, M-PMV	Global inhibition of class E Vps machinery	Garrus et al., 2001; Goila-Gaur et al., 2003; Gottwein et al., 2003; Shehu-Xhilaga et al., 2004; Tanzi et al., 2003; von Schwedler et al., 2003
Tsg101 overexpression	HIV-1	Disrupts ESCRT-I machinery	Goila-Gaur et al., 2003; Shehu-Xhilaga et al., 2004
AIP1/Alix overexpression	EIAV	ND ^a	Strack et al., 2003
CHMP2A ^b overexpression	HIV-1, MLV, Retroviral construct that harbors a YPxL-type L domain	ND ^a	Martin-Serrano et al., 2003a,b; von Schwedler et al., 2003
CHMP3 ^b overexpression	HIV-1	ND ^a	Strack et al., 2003; von Schwedler et al., 2003
CHMP4 ^b (4A-, 4B-, and 4C) overexpression	HIV-1	ND ^a	Gottwein et al., 2003; Martin-Serrano et al., 2003a, b; Strack et al., 2003; Tanzi et al., 2003; von Schwedler et al., 2003
CHMP5 ^b overexpression	Retroviral constructs that harbor PTAP- or YPxL-type L domains	ND ^a	Martin-Serrano et al., 2003a,b

^a Not defined.

^b The protein was fused to either RFP, CFP, DsRed, or YFP; upon expression it may function in a dominant negative manner.

conclusion that TSG-5' inhibition is specific for P(T/S)AP was obtained from the observation that while WT EIAV is unaffected by TSG-5', a mutant EIAV in which the YPDL L domain motif is substituted with PTAP is sensitive to TSG-5' inhibition (Shehu-Xhilaga et al., 2004). Consistent with a role for the HTLV-I P(T/S)AP motif in virus release, HTLV-I budding is also inhibited by overexpression of TSG-5' (Bouamr et al., 2003).

Overexpression of full-length Tsg101 also inhibits HIV-1 release (Goff et al., 2003; Goila-Gaur et al., 2003), as does overexpression of the C-terminal half of the protein (TSG-3') (Goila-Gaur et al., 2003) (Table 1). In contrast to the inhibition induced by TSG-5', which is P(T/S)AP-specific, the release defect induced by TSG-3' does not require a direct association with Gag and is more global, as other retroviruses

(e.g., MLV) are also strongly inhibited (Goila-Gaur et al., 2003; Shehu-Xhilaga et al., 2004). Overexpression of full-length Tsg101 induces a swollen endosomal phenotype that disrupts the sorting of the epidermal growth factor receptor (Goila-Gaur et al., 2003). Interestingly, TSG-3' induces the formation of very large swollen vacuoles or aggregates, the biogenesis of which remains to be determined (Goila-Gaur et al., 2003). This issue is of considerable interest since TSG-3' very potently inhibits P(T/S)AP- and PPxY-dependent release but does not disrupt the budding and release of WT EIAV (Shehu-Xhilaga et al., 2004). By an unknown mechanism, cellular Ub is sequestered within TSG-3'-induced structures (Marc Johnson, personal communication). Depletion of free Ub by both proteasome inhibitors and TSG-3' expression could perhaps explain the observation that both

of these budding inhibitors display a parallel pattern of activity against retroviral release; i.e., both inhibit P(T/S)AP- and PPxY-dependent release but are inactive against YPxL-dependent budding (Shehu-Xhilaga et al., 2004).

Consistent with the hypothesis that Tsg101 binding serves to connect HIV-1 Gag to the ESCRT machinery, perturbation of not only Tsg101 but also Vps28 inhibits particle release (Martin-Serrano et al., 2003b).

4.3. PPxY L domains interact with Nedd4-related E3 Ub ligases

PPxY motifs in cellular proteins have long been known to interact with so-called WW domains, which are approximately 40 amino acids in length and contain two Trp residues spaced 20–22 residues apart (Sudol, 1996; Sudol and Hunter, 2000). Indeed, the L domain of RSV was reported to interact *in vitro* with the WW domain of Yap (for Yes-associated protein) (Garnier et al., 1996); however, the significance of this interaction for RSV release remains unclear.

A number of studies have identified Nedd4 (for neuronal precursor cell-expressed developmentally down-regulated-4) and related proteins as the predominant host factors interacting with PPxY-containing retroviral Gag proteins. Nedd4 family members are E3 Ub ligases that typically harbor N-terminal, calcium-inducible membrane binding (“C2”) domains, multiple centrally located WW domains, and a C-terminal HECT domain (for homologous to E6 C-terminus) that possesses the Ub ligase activity (Harvey and Kumar, 1999) (Fig. 3). The internal region comprising the WW domains determines target protein specificity.

Screening of a chicken cDNA expression library with a biotinylated RSV p2b peptide identified a protein termed LDI-1 (for L domain-interacting protein 1) that bound the p2b peptide in a PPxY-dependent manner (Kikonyogo et al., 2001). LDI-1 bears homology to the Nedd4-family of proteins, though it lacks the catalytic HECT domain. Overexpression of an LDI-1 fragment spanning the WW domains inhibited RSV budding in a dominant negative manner (Kikonyogo et al., 2001) (Table 1). In coimmunoprecipitation experiments, M-PMV Gag was found to bind a protein termed BUL1 (for budding-associated ubiquitin ligase 1) that was also homologous to Nedd4 and interacted with the PPxY motif of M-PMV Gag (Yasuda et al., 2002). Overexpression of BUL1 significantly enhanced virus budding whereas overexpression of Nedd4 or a truncated BUL1 peptide that retained only the WW domains modestly inhibited M-PMV release. HTLV-I Gag, which contains both PPPY and PTAP L domain motifs, was reported to interact with Nedd4 (Blot et al., 2004; Bouamr et al., 2003; Sakurai et al., 2004) and the Nedd4-related protein WWP1 (Heidecker et al., 2004). Nedd4 or WWP1 fragments inhibited HTLV-I release (Bouamr et al., 2003; Heidecker et al., 2004). Nedd4 family members may also function in the release of the rhabdoviruses and filoviruses, as the matrix proteins of these viruses, which contain PPxY L domains, have been reported to interact with Nedd4 or re-

lated proteins (Harty et al., 1999, 2001, 2000; Timmins et al., 2003) (see chapters by Jasenosky and Kawaoka; and Jayakar et al.).

Since Nedd4 and its relatives regulate the cell-surface expression of membrane proteins (e.g., the sodium epithelial channel protein ENaC) via the MVB pathway, the interaction of PPxY L domains with Nedd4 family members may be functionally relevant to virus budding. It is noteworthy that Rsp5, the yeast homolog of Nedd4, plays a key role in cargo ubiquitylation (Katzmann et al., 2004). Nedd4-mediated Gag ubiquitylation could therefore promote the entry of PPxY-containing Gag into the MVB sorting pathway. However, the mechanism by which PPxY association with Nedd4-related Ub ligases would stimulate particle release remains to be defined. It has been observed that HTLV-1-mutant Gag lacking the PPxY motif accumulates at the plasma membrane, whereas P(T/S)AP-defective Gag accumulates in an MVB compartment. These results prompted the authors to propose that Gag first associates with Nedd4 at the plasma membrane and subsequently binds Tsg101 in the MVB (Blot et al., 2004).

4.4. YPxL and related L domains interact with AIP1/Alix

A number of proteins that harbor Tyr-based (YxxL) sorting signals bind the medium chains of AP-1 and AP-2 clathrin-associated adapter protein complexes (Ohno et al., 1995; Trowbridge et al., 1993). This raised the possibility that the YPDL L domain motif of EIAV functions by interacting with adapter protein complexes. Indeed, *in vitro* studies demonstrated that EIAV p9 binds the AP-50 subunit of the AP-2 complex, and p9 and AP-2 colocalize in Gag-expressing cells (Puffer et al., 1998). However, the functional significance of the interaction between EIAV Gag and the AP-2 complex remains to be demonstrated. Recent evidence has pointed to a different cellular factor, AIP1/Alix, as the primary EIAV L domain partner.

EIAV is distinct from the majority of retroviruses both in terms of its unique L domain and the insensitivity of its budding to proteasome inhibitors. Despite these differences, the release of EIAV particles is also blocked by a dominant negative Vps4 mutant (Martin-Serrano et al., 2003a; Shehu-Xhilaga et al., 2004; Strack et al., 2003; Tanzi et al., 2003; von Schwedler et al., 2003) suggesting that this non-primate lentivirus also utilizes the MVB sorting machinery for its release. The observation that the *Aspergillus* homolog of Vps31 recognizes a YPxL/I core motif led to the suggestion that the mammalian homolog of Vps31, AIP1/Alix (Missotten et al., 1999; Vito et al., 1999) might be the p9-interacting host factor (Vincent et al., 2003) (Fig. 3). *In vitro* studies confirmed the prediction that AIP1/Alix interacts with the EIAV p9 L domain in a YPxL-dependent manner (Martin-Serrano et al., 2003a; Strack et al., 2003; von Schwedler et al., 2003). More unexpected was the observation that AIP1 also interacts with HIV-1 p6 via the sequence LYPLxxL (Strack et al., 2003). This motif, which bears some homology with the LYPxL se-

quence in EIAV, is located downstream of P(T/S)AP (Fig. 1) (Strack et al., 2003).

The role of AIP1/Alix in retrovirus release has been probed using a variety of approaches, including depletion with siRNA and overexpression of WT or truncated forms of the protein. In addition, full-length AIP1/Alix or truncated mutants have been fused to green fluorescence protein (GFP) derivatives, as this approach can generate dominant negative forms of class E Vps proteins (Howard et al., 2001). Depletion of endogenous AIP1/Alix by siRNA or overexpression of the C-terminal, YPX_L-binding domain of AIP1/Alix inhibits EIAV p9-dependent virus release; i.e., in the context of coexpression of p6-deleted HIV-1 Gag with HIV-1 Gag fused to EIAV p9 (Martin-Serrano et al., 2003a) (Table 1). In contrast, AIP1/Alix depletion or C-terminal fragment overexpression has relatively little effect when release is P(T/S)AP-dependent (Martin-Serrano et al., 2003a). Dominant negative derivatives of AIP1/Alix inhibit the budding of both HIV-1 and EIAV (Strack et al., 2003), though the effect on HIV-1 may be due to global perturbation of the MVB sorting machinery, as reported for overexpression of full-length Tsg101 (Goila-Gaur et al., 2003). Current data suggest that AIP1/Alix is a major player in EIAV (YPX_L-dependent) particle release, whereas the role of this factor in HIV-1 budding remains to be defined.

Dominant negative forms of several of the ESCRT-III components (the CHMP proteins) also block HIV-1 budding (Martin-Serrano et al., 2003a; Strack et al., 2003; von Schwedler et al., 2003) (Table 1). As with AIP1/Alix, the effect of CHMP dominant-negative proteins on HIV-1 release is most likely mediated through a general disruption of the class E Vps machinery.

5. The targeting of retrovirus release

While C-type retroviruses and lentiviruses have long been observed to bud from the plasma membrane, it is becoming clear that, at least under certain circumstances, retrovirus assembly and budding can take place in the MVB. EM observations provided evidence for budding of HIV-1 into intracellular vesicles in primary monocyte-derived macrophages (Orenstein et al., 1988), and it has been determined recently that this virus-positive intracellular compartment expresses late endosomal or MVB markers (Gould et al., 2003; Nguyen et al., 2003; Nydegger et al., 2003; Ono and Freed, 2004a; Pelchen-Matthews et al., 2003; Raposo et al., 2002; Sherer et al., 2003). HIV-1 Gag may target the MVB in other cell types as well (Nydegger et al., 2003; Sherer et al., 2003). HTLV-1 can also assemble in an intracellular compartment (Le Blanc et al., 2002a), perhaps the MVB (Blot et al., 2004; Wang et al., 2004). Subsequent to the budding of virus particles into the lumen of the MVB, virus release from the cell likely occurs via the exosome pathway, in which MVBs traffic to, and fuse with, the plasma membrane (Denzer et al., 2000; Stoorvogel et al., 2002) (Fig. 4). The release of virus-laden MVBs may

occur directionally at points of contact between macrophages and T-cells, thereby facilitating cell–cell transmission.

It remains to be determined what controls Gag trafficking to the plasma membrane vs. the MVB. Given the association between L domains and MVB machinery, it would seem plausible that L domains regulate targeting to the MVB. However, recent analysis indicates that p6-deleted HIV-1 Gag mutants still assemble in the MVB in primary macrophages (Ono and Freed, 2004a). Interestingly, p6-deleted Gag displays a tethering defect at the MVB membrane in macrophages analogous to that observed for L domain mutants at the plasma membrane in HeLa cells (Demirov et al., 2002b) (Fig. 2). Recent evidence suggests that specific phosphoinositides may play a role in regulating Gag targeting (Ono et al., 2004) and a di-Leu motif in HIV-1 CA has been implicated in the trafficking of Gag to the MVB (Lindwasser and Resh, 2004).

The assembly of HIV-1, other retroviruses, and a number of non-retroviral enveloped viruses is thought to occur in plasma membrane microdomains known as lipid rafts that are enriched in cholesterol and glycosphingolipids. Association of viral structural proteins with lipid rafts may promote membrane binding and protein–protein interactions during assembly and may also enhance cell–cell transmission and increase the infectivity of cell-free virus. The role of lipid rafts in enveloped virus replication is reviewed in detail elsewhere (Briggs et al., 2003; Nayak and Barman, 2002; Ono and Freed, 2004b; Suomalainen, 2002).

6. Non-Gag determinants of retrovirus release

While in general Gag is the only retroviral protein required for efficient particle release, Gag targeting and virus release can be influenced by the expression of other viral proteins. In the case of the spumaretroviruses, expression of Gag alone results in the retention of particles in the cytosol. Efficient budding and extracellular release of these particles requires Env expression (Fischer et al., 1998). The M-PMV Env also appears to play a role in directing particle release from the plasma membrane. In this system, Gag assembles into immature capsids in a pericentriolar region of the cytoplasm. Subsequent trafficking of the capsids to the plasma membrane is promoted by Env (Sfakianos and Hunter, 2003; Sfakianos et al., 2003). Although, HIV-1 Env does not appear to stimulate particle release efficiency, in some cases it plays a role in Gag targeting. In polarized epithelial cells, Gag VLPs are released from both apical and basolateral surfaces. If Env is expressed, particle release occurs predominantly from the basolateral surface; this targeted release requires a basolateral targeting sequence in the cytoplasmic tail of the transmembrane glycoprotein gp41 (Lodge et al., 1994, 1997; Owens et al., 1991). Although, the physiological relevance of the polarized epithelial cell system for HIV-1 replication is uncertain, it has been suggested that Env may perform a similar role in directing Gag assembly and release in lymphocytes (Deschambeault et al., 1999). MLV particles have

been reported to assemble on endosomal membranes and then traffic to the plasma membrane in an Env-dependent fashion (Basyuk et al., 2003). In addition to Env, another retroviral protein, Vpu of HIV-1, modulates particle production. Expression of Vpu stimulates virion release several-fold in human cell types (Bour and Strebel, 2003) and Vpu-deficient particles accumulate intracellularly and at the plasma membrane (Klimkait et al., 1990). The role of Vpu in promoting particle production is distinct from that of p6 (Schwartz et al., 1996) and may involve the countering of a human-cell-specific restriction to virus release (Varthakavi et al., 2003). HIV-2 does not encode a Vpu protein; interestingly, however, it has been reported that the HIV-2 Env protein is able to perform a Vpu-like role in enhancing particle production (Bour et al., 1996).

7. A model for retrovirus budding

Abundant evidence now supports the hypothesis that L domains function by usurping cellular machinery normally used to catalyze the budding of cargo-laden vesicles into the MVB (Fig. 4). The observation that budding promoted by all three classes of retroviral L domains is strongly inhibited by dominant negative Vps4 demonstrates the widespread importance of an intact class E Vps sorting machinery in retrovirus release. It is also clear that the entry point into the MVB pathway is determined by the specific partners with which the different classes of L domains interact. For viruses like HIV-1 whose release is mediated primarily by a P(T/S)AP motif, association with the MVB machinery takes place at the level of ESCRT-I through a direct interaction with Tsg101. The release of HIV-1 is therefore impaired by proteasome inhibitors and by disruption of ESCRT-I (e.g., Tsg101 depletion) or competition with the Gag-Tsg101 interaction (e.g., TSG-5' overexpression). YPxL-dependence results in the recruitment of MVB machinery at a point downstream of ESCRT-I and -II through interaction with AIP1/Alix. The YPxL-dependent EIAV is thus resistant to proteasome inhibitors and TSG-3' overexpression but is sensitive to disruption of AIP1/Alix, ESCRT-III components, or Vps4 activity. The point of entry of PPxY-dependent viruses is less well defined, as the mechanism by which interaction between PPxY and putative Nedd4-related partners would provide access to the class E Vps machinery remains to be elucidated. However, current data suggest that PPxY-dependent retroviruses enter the MVB sorting pathway upstream of EIAV since the former are, like HIV-1, sensitive to proteasome inhibitors and TSG-3' overexpression. PPxY utilization also appears to bypass ESCRT-I, as viruses like MLV are insensitive to Tsg101 siRNA or TSG-5' (Demirov et al., 2002a; Garrus et al., 2001). Due to L-domain-specific interactions between retroviral Gag and host proteins, the sensitivity of L domains to release inhibitors can be controlled by L domain exchange (Shehu-Xhilaga et al., 2004). The observation that in some cases L domain mutations induce an accumulation of Gag in electron-

dense, membrane-associated patches suggests that L domains may be involved in both bud formation and particle release.

8. Concluding remarks

A remarkable convergence of cell biology and virology in the past several years has provided detailed insights into the mechanism by which host proteins are sorted from the endocytic or biosynthetic pathway into the MVB, and the fascinating manner in which a number of enveloped viruses, including the majority of retroviruses, have taken advantage of this pathway to promote their release from the cell.

Despite the rapid progress in these overlapping fields of research, many key questions remain unanswered. (1) Which host factor(s) catalyze the final “pinching-off” step in virus release or ILV budding? In general, perturbation of class E Vps machinery results in the formation of enlarged, aberrant endosomes rather than in the generation of ILVs that remain tethered to the endosomal membrane. The class E Vps machinery implicated thus far in L domain activity may well set the stage for efficient release, but the factor(s) that catalyze the “pinching-off” step are as yet unidentified. It is tempting to speculate that endophilins or other enzymes with lipid-modifying activity could provide this function. It is also interesting to note that a pinching-off defect of ILVs at the luminal side of endosomes is observed in cells treated with wortmannin (Bright et al., 2001; Fernandez-Borja et al., 1999; Futter et al., 2001). This compound inhibits phosphatidylinositol 3-kinase (Vps34) activity thereby preventing phosphatidylinositol 3-phosphate [PI(3)P] generation through the phosphorylation of phosphatidylinositol. These results suggest a role for PI(3)P in ILV budding. However, the pleiotropic effects of wortmannin treatment, and the involvement of PI(3)P in multiple cellular pathways, makes clear interpretation of these observations difficult. Also noteworthy is the recently described connection between AIP1 and LBPA, an MVB-localized lipid that may promote membrane curvature (Matsuo et al., 2004). (2) Why have retroviruses evolved several classes of L domain? Since it appears that L domains are largely exchangeable, one might imagine that all retroviruses could be released efficiently through the use of a single, conserved L domain. The answer to this question perhaps lies in elucidating the function of L domains in specific cell types naturally infected by retroviruses; it follows that more studies on L domain activity should be performed in physiologically relevant cell types. (3) Why do so many retroviruses harbor multiple L domains, in particular P(T/S)AP and PPxY? Again, it seems likely that L domain redundancy or co-operativity might provide for optimal release efficiency in cell types naturally infected in vivo, perhaps under conditions of variable or limiting concentrations of L domain-interacting host factors. It is also possible that L domain evolution could be driven by competition between functional L-domain-interacting factors (e.g., Tsg101, AIP1, etc.) and related cellular proteins. For example, a num-

ber of Nedd4 family members have been identified (Sudol, 1996) and a protein known as UEV3 contains a UEV domain with significant homology to the Gag-binding domain of Tsg101 (Kloor et al., 2002). In addition, as mentioned above, L domains may perform multiple, sequential roles in the budding and release pathway. (4) What is the role of Ub in virus release and why do proteasome inhibitors disrupt budding? While Ub plays a central role in the sorting of many cargo proteins into the MVB pathway, a requirement for Gag ubiquitylation remains to be established. Proteasome inhibitors may block virus release by affecting the ubiquitylation, localization, or sorting of host factor(s), rather than by directly impacting Gag ubiquitylation. It is possible that the mechanism by which proteasome inhibitors disrupt virus release may vary between retroviruses and between L domain classes. (5) How do Gag proteins bearing PPxY L domains access the MVB pathway? While many studies have implicated Nedd4 or related proteins as the physiological partners for PPxY, the mechanism by which Nedd4 interaction would facilitate an association with class E Vps machinery remains to be defined. Perhaps relevant to this question is the recent observation that the yeast homolog of Nedd4 acts as the E3 Ub ligase that ubiquitylates MVB cargo (Katzmann et al., 2004). The recruitment of Nedd4 (or related proteins) by Gag could catalyze the ubiquitylation of Gag itself or of a cellular factor involved in the budding process. It is also possible that factors in addition to Nedd4 or its relatives might serve as ligands for PPxY motifs. (6) What regulates whether virus release occurs via the exosome pathway or directly from the plasma membrane? As mentioned above, the targeting of virus assembly does not appear, at least in the case of HIV-1, to be determined by the L domain (Ono and Freed, 2004a). Phosphoinositides are potential candidates as regulators of Gag targeting, as these lipids have been widely implicated in the trafficking of a number of cellular membrane-associated proteins (Simonsen et al., 2001). Indeed, recent evidence implicates phosphatidylinositol-(4,5)-bisphosphate in HIV-1 Gag targeting (Ono et al., 2004).

The answers to these and related questions are certain to increase our understanding of virus budding and release, as well as provide insights into the corresponding cellular processes of endosomal sorting and class E Vps protein function. These topics are of more than academic interest, as virus release machinery may constitute a novel target for the development of anti-retroviral therapies. While global disruption of the class E Vps machinery would likely not be well tolerated, blocking interactions between L domains and their cellular partners may be possible without overwhelming toxicity. This notion gains support from the observation that over-expression of TSG-5' does not appear to disrupt the cellular endosomal sorting machinery, at least in a qualitative sense (Goila-Gaur et al., 2003), and from the finding that cell lines stably expressing TSG-5' are readily obtainable (Goila-Gaur, Shehu-Xhilaga, and Freed, unpublished). While TSG-5' itself is unlikely to be useful as a therapeutic, small molecules that act in an analogous manner (by blocking the interaction be-

tween p6 and Tsg101) could in theory be clinically effective. The apparent use of Tsg101 by human pathogens as diverse as HIV-1, HTLV-1, and Ebola further suggests that inhibitors that block L domain function could be engineered to display broad antiviral activities.

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