Avian Sarcoma Virus and Human Immunodeficiency Virus, Type 1 Use Different Subsets of ESCRT Proteins to Facilitate the Budding Process*

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Members of the Nedd4 family of E3 ubiquitin ligases bind the L domain in avian sarcoma virus (ASV) Gag and facilitate viral particle release. Translational fusion of ASV Gag with an L domain deletion (Δp2b) to proteins that comprise ESCRT-I, -II, and -III (the endocytic sorting complexes required for transport) rescued both Gag ubiquitination and particle release from cells. The ESCRT-I factors Vps37C or Tsg101 were more effective in rescue of Gag/Δp2b budding than the ESCRT-II factor Eap20 or the ESCRT-III component CHMP6. Thus ESCRT components can substitute for Nedd4 family members in ASV Gag release. Unlike wild type, ASV Gag/Δp2b-ESCRT chimeras failed to co-immunoprecipitate with co-expressed hemagglutinin-tagged Nedd4, indicating that Nedd4 was not stably associated with these Gag fusions. Release of the Gag-ESCRT-I or -II fusions was inhibited by a dominant negative mutant of Vps4 ATPase similar to wild type ASV Gag. In contrast to ASV Gag, HIV-1 Gag containing an L domain inactivating mutation (P7L) was efficiently rescued by fusion to a component of ESCRT-III (Chmp6) but not ESCRT-II (Eap20). Depletion of the endogenous pool of Eap20 (ESCRT-II) had little effect on HIV-1 Gag release but blocked ASV Gag release. In contrast, depletion of the endogenous pool of Vps37C (ESCRT-I) had little effect on ASV but blocked HIV-1 Gag release. Furthermore, an N-terminal fragment of Chmp6 inhibited both HIV-1 and ASV Gag release in a dominant negative manner. Taken together, these results indicate that ASV and HIV-1 Gag utilize different combinations of ESCRT proteins to facilitate the budding process, although they share some common elements.

Retroviruses, and many other enveloped viruses, evolved mechanisms to exploit components of the endocytic sorting pathway to bud from cells efficiently. The retroviral Gag precursor polyprotein contains the major structural components of the virus, including late assembly domains that function as docking sites for host cell factors that promote the release of virus-like particles (VLPs) from the plasma membrane (1–7). Although distinct classes of L domain sequences exist among retroviruses (with core motifs of PTAP, PPXY, and YPXL), each functions as a binding site to recruit different components of the vacuolar protein sorting (Vps) machinery. Class E Vps proteins function in the process of sorting cargo proteins into the luminal vesicles of multivesicular bodies (MVBs). MVBs are carrier vesicles that deliver cargo designated for lysosomal degradation from early to late endosomes (8). Functionally conserved from yeast to mammalian cells, most class E Vps proteins are organized into high molecular weight cytoplasmic complexes called ESCRT (endocytic sorting complexes required for transport) -I, -II, and -III. Although the mechanistic details of MVB biogenesis remain poorly characterized, current studies suggest that the ESCRT complexes are sequentially recruited to endosomal compartments to remodel and invaginate the limiting endosomal membrane and sort ubiquitinated protein cargo into luminal vesicles. In the last defined step, the ESCRT-III complex recruits an AAA ATPase, Vps4, to catalyze the dissociation of the ESCRT complexes from the endosomal compartment. This facilitates scission of the vesicle from the limiting membrane (for reviews, see Refs. 9–13).

The PTAP and PPXY core motifs in HIV-1 and ASV Gag, respectively, are functionally exchangeable (14). This, together with the finding that release of both viruses from cells is blocked by the catalytically inactive Vps4 E228Q enzyme, underlines usage of a common budding process dependent on the ESCRT machinery (15, 16). However, the molecular basis for this functional exchangeability remains unclear because the mechanism by which ASV Gag accesses components of the ESCRT machinery is not known. The PTAP motif, located in the p6 region of HIV-1 Gag, binds to the class E Vps protein, Tsg101 (5). The p6 region of HIV-1 Gag also contains a secondary L domain motif (YPXL) that recruits another class E Vps protein, AIP1/Alix (17). AIP1 acts as a bridging factor to link ESCRT-I to the ESCRT-III complex. The PPXY motif, located in the p2b region of ASV Gag, binds to the Nedd4 family of E3 HECT ubiquitin

* The abbreviations used are: VLP, virus-like particle; Vps, vacuolar protein sorting; HIV-1, human immunodeficiency virus, type 1; ASV, avian sarcoma virus; MVB, multivesicular bodies; E3, ubiquitin-protein isopeptide ligase; HA, hemagglutinin; siRNA, small interfering RNA; GFP, green fluorescent protein; mAb, monoclonal antibody; FL, full length.

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ligases. Unlike Tsg101, Nedd4 is not a class E Vps protein in that its function is not required for MVB biogenesis. However, like AIP1, Nedd4 binds Tsg101 (15) and therefore may link ASV Gag to the ESCRT machinery. Co-expression of catalytically inactive Nedd4 with a point mutation in its HECT domain inhibits the release of ASV Gag, providing strong evidence that ubiquitin signaling plays an important role in Gag release (18). Although the specific role of ubiquitin in retrovirus budding remains unclear, it may potentially function as a sorting signal to facilitate the interaction between Gag and ubiquitin-binding domains in some of the factors of the ESCRT machinery. Recent studies also demonstrate that overexpression of Nedd4L rescues the budding defect caused by the deletion of the L domain in HIV-1 Gag. Although these findings implicate Nedd4 activity in particle release, the precise role of Nedd4L in wild type HIV-1 budding remains unclear (19, 20).

In this report, we demonstrate the ability of different ESCRT proteins covalently linked to ASV Gag/Δ2b or HIV-1 Gag P7L, both of which have L domain mutations (see Fig. 1), to support VLP release. We observe that ESCRT-I, -II, and -III proteins rescue ASV Gag budding with declining efficiencies, respectively. We further show that a productive budding pathway correlates with the restoration of ubiquitin modification of chimeric Gag. Interestingly, tethering the ESCRT-II protein Eap20 to the C terminus of Gag complements the L domain mutation of ASV Gag/Δ2b but not HIV-1 Gag P7L. This suggests that, unlike HIV-1 Gag, ASV Gag requires a functional ESCRT-II complex for release. Accordingly, we observe that siRNA-mediated depletion of Eap20 in 293/E cells significantly inhibits the release of ASV but not HIV-1 Gag. tethering the ESCRT-I protein, Vps37C, to ASV Gag/Δ2b also rescues budding and ubiquitination. However, depletion of the endogenous pool of Vps37C has no affect on release of ASV Gag but blocked the release of HIV-1 Gag. The biochemical analysis presented in this manuscript, together with our previous findings, indicate that ASV and HIV-1 Gag use parallel pathways dependent on different components of the ESCRT machinery to bud from cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All ASV Gag expression constructs were based on plasmid 2036 as previously described (4, 18). To create the ASV Gag/Δp2b-ESCRT and HIV Gag/P7L-ESCRT fusion constructs, an HpaI site was introduced in frame at the 3′ end of the Gag coding region by standard PCR techniques. Similarly an HpaI site was added to the 5′ end of the coding region of all ESCRT proteins. The fusion constructs were then cloned into p2036. Vps37C was PCR-amplified from pCR3.1/YFP-Vps37C, a generous gift from Scott Eastman and Paul Bieniasz (Rockefeller University) (21). pMT123 encodes an HA-tagged ubiquitin ligase. Unlike Tsg101, Nedd4 binds Tsg101 (15) and therefore may link ASV Gag to the ESCRT machinery. Co-expression of catalytically inactive Nedd4 with a point mutation in its HECT domain inhibits the release of ASV Gag, providing strong evidence that ubiquitin signaling plays an important role in Gag release (18). Although the specific role of ubiquitin in retrovirus budding remains unclear, it may potentially function as a sorting signal to facilitate the interaction between Gag and ubiquitin-binding domains in some of the factors of the ESCRT machinery. Recent studies also demonstrate that overexpression of Nedd4L rescues the budding defect caused by the deletion of the L domain in HIV-1 Gag. Although these findings implicate Nedd4 activity in particle release, the precise role of Nedd4L in wild type HIV-1 budding remains unclear (19, 20).

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MA(p19), developed by David Boettiger, was obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences at the University of Iowa (Iowa City, IA). Ubiquitinated forms of Gag were detected with a mouse anti-HA antibody.

siRNA Depletion of Endogenous Vps37C and Eap20 in Cells—Knockdown of YFP-Vps37C and Eap20-FLAG expression was achieved by transfecting the indicated concentrations of Vps37C or Eap20 siRNA into 293/E cells in six-well plates with Lipofectamine RNAiMAX (Invitrogen). Forty-eight hours post-transfection, the 293/E cells were transfected with plasmid DNA encoding either YFP-Vps37C, EAP20-FLAG, ASV Gag, or HIV Gag-GFP with the FuGENE 6 transfection reagent as described above. VLPs and lysate fractions were harvested 24 h later, and the levels of proteins expression were analyzed by quantifying the intensities of the bands obtained in Western blots as described above. The Eap20 siRNA used in this study was originally described by Langelier et al. (23). The control siRNA, a random, nontargeting sequence, was purchased from Dharmacon. The Vps37C siRNA pool consists of a mixture of four siRNA duplexes directed against the coding sequence of human Vps37C. The sense sequence of the siRNA set with the 3’ overhanging UU dinucleotides are as follows: (a) agcagcaccccccguuauua, (b) gcuccacuccgccgauaguu, (c) gccacccggaaauuuguu, and (d) gcgcgccgaccuauauu.

RESULTS

ESCRT Proteins Complement an L domain Deletion when Covalently Linked to the C Terminus of ASV Gag/Δp2b—Previous studies established that covalently linking Hrs (24), or ESCRT-I proteins Tsg101 (25), Vps37B (26), or Vps37C (21) to the C-terminal end of HIV-1 Gag/Δp6 restored the release of VLPs, supporting the view that the PTAP motif functions to recruit ESCRT-I activity to the site of viral particle assembly. Such functional replacement of L domain activity provides a valuable assay to characterize the role of ESCRT complexes in retroviral budding. In this study, we expanded on previous approaches by constructing seven chimeric ASV Gag/Δp2b-ESCRT proteins and monitored their ability to rescue the budding defect caused by an 11 amino acid deletion that includes the PPXY motif in the p2b region of ASV Gag (6, 7) (Fig. 1). Each chimera was transfected into 293/E cells, cell lysate and media fractions were prepared, Gag complexes were recovered, and viral proteins were detected by Western blotting after fractionation by SDS-PAGE, as described under “Detection of Proteins by Western Blotting.” To stabilize expression, ASV Gag/Δp2b-Tsg101 was co-expressed with Vps28-FLAG, and ASV Gag/Δp2b-Eap45 was co-expressed with Eap20-FLAG. Eap45 expression is not stable unless co-expressed with other ESCRT-II proteins (23). Tsg101 expression is greatly enhanced when co-expressed with Vps28 to prevent proteosome-mediated degradation (27). Also, proteolytic processing of Gag is not required for particle release from cells so that all ASV Gag constructs contain a D37S mutation in the protease coding sequence to permit detection of full-length rather than processed Gag proteins on the gels. Efficient release of VLPs into the medium fraction depends upon the p2b region of ASV Gag (Fig. 2, lanes 1 and 2), in agreement with previous observations (6, 7). In this experiment, the amount of Gag/Δp2b in the medium fraction was barely detected (lane 2). As shown in Fig. 2, fusion of Gag/Δp2b to Vps37C (lane 3), Tsg101 (lane 5), Eap20 (lane 6), or Chmp6 (lane 8) rescued budding to different extents. The origin of the faster migrating band observed with Gag/Δp2b-Vps37C fusion in the medium fraction is not known but might reflect protease degradation (Fig. 2, lane 3).

To quantify the extent of rescue, the cells were transfected with wild type Gag, Gag/Δp2b, or each of the fusion constructs

FIGURE 1. Domain organization of ASV and HIV-1 Gag and fusion constructs. ASV and HIV-1 Gag polyproteins are 76 kDa (Pr76Gag) and 55 kDa (Pr55Gag), respectively. The vertical lines represent protease cleavage sites delineating the mature viral proteins. Gag/Δp2b lacks the 11-amino acid p2b region that contains the PPXY L domain motif. All ASV Gag constructs used for this report contain an inactivating D37S substitution in the protease subunit. In the Gag/Δp2b-ESCRT chimera proteins (linked to Gag/Δp2b-Tsg101, -Vps37C, -Vps28, -Eap20, -Eap45, -Chmp4B, or -Chmp6), the ESCRT proteins were fused to the C terminus of Gag. A GFP was fused to the C terminus of HIV-1 Gag. An L domain defective HIV-1 Gag-GFP construct has a point mutation, p7L, in its PTAP sequence in p6. The Eap20, Eap45, Chmp6, and Chmp4B ESCRT proteins were fused to the C terminus as indicated. Myr is a myristylate found on the N terminus of Pr55Gag, Ac is acetate found on the N terminus of Pr76Gag.

FIGURE 2. ESCRT proteins covalently linked to ASV Gag/Δp2b rescue the budding defect caused by the L domain deletion. The cells (293E) were transfected with plasmid encoding wild type, L domain-deleted (Δp2b), and C-terminal fusions of various ESCRT proteins to ASV Gag/Δp2b as indicated and as described under “Experimental Procedures.” VLPs were harvested from the media fractions by centrifugation through a 20% sucrose cushion at 100,000 × g for 1 h to form a pellet. Gag constructs from cell lysates were immunoprecipitated with an anti-ASV rabbit polyclonal antisera. Gag proteins from each fraction were resolved by SDS/PAGE and detected by Western blotting using a monoclonal antisera directed at the ASV mAb MA(p19) protein. The top panel shows cellular expression levels of wild type ASV Gag (lane 1), ASV Gag/Δp2b (lane 2), and ASV Gag/Δp2b fused to Vps37C (lane 3), to Vps28 (lane 4), to Tsg101 (lane 5), to Eap20 (lane 6), to Eap45 (lane 7), to Chmp6 (lane 8), and to Chmp4B (lane 9). The bottom panel shows VLPs released into the media fractions. Note: to stabilize expression, ASV Gag/Δp2b-Tsg101 was co-expressed with Vps28-FLAG, and ASV Gag/Δp2b-Eap45 was co-expressed with Eap20-FLAG. The arrows show full-length Gag or Gag fusion proteins as estimated by migration through the denaturing gels and comparison with proteins of known molecular weights. 
and labeled with \(^{35}\text{S}\)Met/Cys as described under "Experimental Procedures." Radiolabeled Gag and Gag fusions were immunoprecipitated from the cell lysate and media fractions and fractionated on denaturing polyacrylamide gels, and radioactivity bands were quantified. Setting the budding efficiency of wild type Gag to 1 and normalizing for the amount of total full-length Gag or Gag fusion expressed in the lysate and media fractions, the relative release efficiencies of the ESCRT chimeras was averaged from two separate experiments. When fused to Gag/Δp2b, the ESCRT-I proteins Vps37C and Tsg101 rescued VLP release to ~65 and 45%, respectively, relative to wild type Gag. The ESCRT-II protein, Eap20, restored VLP release with a budding efficiency of ~25%. Lastly, we observed a 13% rescue of VLP budding for the Chmp6 fusion relative to wild type Gag. We also detected an amount of Gag/Δp2b-Tsg101, with Gag/Δp2b-Chmp6 of ~4% by this protocol, which is not sufficient to differentiate from Gag/Δp2b. Thus the Western and radiolabeled analyses yielded similar results. Of the seven ESCRT proteins fused to Gag/Δp2b, Vps28 (ESCRT-I), Eap45 (ESCRT-II), and Chmp4B (ESCRT-III) failed to complement the L domain deletion (lanes 4, 7, and 9) when fused to Gag/Δp2b so that their role in the release process could not be analyzed by this approach. The finding that Eap20, co-expressed with Gag/Δp2b-Eap45, did not rescue Gag release compared with Eap20 covalently linked to Gag/Δp2b suggests that the deletion of the Nedd4 PY-binding motif from Gag removes the capacity of Gag to recruit the necessary ESCRT complexes for the budding process.

**ASV Gag/Δp2b-ESCRT Chimeras Reconstitute a Vps4-dependent Budding Pathway**—We next verified whether the ESCRT-I and -II chimeras utilized the same endocytic sorting machinery for budding as wild type Gag by testing sensitivity to Vps4, a global regulator of the MVB machinery. We co-transfected various Gag/Δp2b-ESCRT fusion constructs with DNA encoding Vps4 E228Q, a catalytically inactive form of Vps4 previously shown to suppress the release of HIV-1 and ASV particles in a dominant negative fashion (2, 16). The data presented in Fig. 3 demonstrates that the release of the Gag fusions to -Tsg101, -Vps37C, and -Eap20, respectively, were subject to dominant negative inhibition by co-expression with Vps4 E228Q in a dose-dependent manner (panels B-D). Quantification of the Gag signals confirms that Vps4 E228Q significantly reduces particle release. The Vps4 E228Q protein is stably expressed in the lysate fraction in each case, as detected by Western blotting (data not shown). From data presented in Figs. 2 and 3, we conclude that ESCRT proteins coupled to Gag/Δp2b allow chimeric Gag to access the endocytic sorting machinery used by wild type.

**Ubiquitination of Gag Is Restored to Gag-ESCRT Chimera That Bud**—The role of ubiquitin remains an important, if yet unclear, feature of retrovirus budding (18, 27–31). Proteasome inhibitors, which deplete free ubiquitin in cells, block PTAP- and PXXY-dependent VLP release (32) and ubiquitination of ASV Gag is dependent upon an intact L domain PY motif (18). Additionally, mutating the lysine residues (the acceptor sites for ubiquitin modification) in close proximity to the HIV and ASV L domains causes a late budding defect (33, 34). We therefore examined whether the ESCRT chimeras restored ubiquitination of Gag as part of a functional budding process. 293/E cells were co-transfected with a vector expressing HA-tagged ubiquitin and expression plasmids encoding wild type Gag, Gag/Δp2b, or ESCRT chimeras. HA-tagged ubiquitin permits enhanced detection of ubiquitinated Gag using an anti-HA antibody. Gag detected in the lysate fraction is both mono- and polyubiquitinated (18). Fig. 4 shows the L domain dependence of Gag ubiquitination, as we detect a greater fraction of monoubiquitinated wild type Gag in the lysate fraction relative to ASV Gag/Δp2b (lanes 1 and 2, upper panel). Interestingly, Gag/Δp2b-Eap20 (lane 7) and Gag/Δp2b-Vps37C (lane 3) induced robust ubiquitination of Gag in the lysate fraction despite the absence of the Nedd4 binding site. We also detected a strong ubiquitination signal for Gag/Δp2b-Tsg101 (lane 6). This pattern of polyubiquitination is similar to Tal-mediated ubiquitination observed with overexpressed Tsg101 (27). In contrast, we did not detect ubiquitination of the Gag/Δp2b-Chmp6...
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**FIGURE 5.** Ubiquitination status of ASV Gag and ESCRT chimeras in medium fractions. ASV Gag, Gag/Δp2b, or Gag/Δp2b-Tsg101, -Chmp6, -Vps37C, and -Eap20 were co-expressed in 293/E cells with the pMT123 vector encoding HA-Ub. VLPs were harvested from medium fractions as described under “Experimental Procedures.” Ubiquitinated forms of Gag were detected by Western blotting with an anti-HA antibody, and VLP release was confirmed by reprobing membrane with an anti-ASV antibody. VLPs were collected from cells transfected with wild type Gag expression vector (lane 1), with Gag/Δp2b (lane 2), with Gag/Δp2b-Tsg101 (lane 3), -Chmp6 (lane 4), -Vps37C (lane 5), and -Eap20 (lane 6). The arrows indicate monoubiquitinated forms of Gag or Gag fusions as in the legend to Fig. 2. Separation between blots indicates grouping of gels to form figure. Where necessary, lanes from a single blot with same exposure were rearranged as indicated by dividing lines.

fusion (lane 4), even though the protein was expressed and stable in the cell lysate fraction. Nor did we detect ubiquitination of Gag/Δp2b-Chmp4B, -Eap45, or -Vps28 fusions (lanes 5, 8, and 9).

We previously demonstrated that either monoubiquitinated ASV Gag is selectively incorporated into VLPs from the pool of ubiquitin-modified Gag in the cytoplasm or polyubiquitinated modified Gag is excluded (Ref. 18 and Fig. 5, lane 1). Similarly, monoubiquitinated forms of ASV Gag/Δp2b-Tsg101 or -Eap20 also were detected in VLPs, although to a lesser extent than wild type Gag (Fig. 5, lanes 3 and 6). In the case of Δp2b/Gag-Vps37C, both the full-length (indicated by the arrow) and the faster migrating forms were monoubiquitinated (lane 5). Because the Gag/Δp2b-Chmp6 fusion was not ubiquitinated in the lysate fraction, we did not detect ubiquitinated forms in the medium fraction, although expression of the fusion protein in VLPs could be detected by Western blotting with an anti-ASV serum (lane 4). Because of the poor ubiquitination in the lysate fraction of Gag/Δp2b-Vps28, -Eap45, and -Chmp4B, we did not detect ubiquitination in the medium fraction (data not shown).

The finding that the Gag/Δp2b-Chmp6 construct achieves partial VLP budding (Fig. 2) in the absence of ubiquitin modification suggests that ubiquitin loses its functional role toward the later stages of the ESCRT pathway.

**FIGURE 6.** Nedd4 does not co-immunoprecipitate with ASV Gag/Δp2b and ASV Gag/Δp2b-ESCRT fusions. HA-tagged Nedd4 (LDI-1 FL) was co-expressed in 293E cells with wild type (lane 1), Gag/Δp2b (lane 2), and Gag/Δp2b-ESCRT fusions (lanes 3–9 as indicated), and HA-Nedd4 was immunoprecipitated from the lysate fraction with an anti-HA antibody as described under “Experimental Procedures.” The proteins in the immunoprecipitates were resolved by SDS-PAGE, and Nedd4 and Gag proteins were detected by Western blotting using anti-HA (lower panel) and anti-ASV (upper panel) rabbit polyclonal serum, respectively.

We co-expressed wild type, Gag/Δp2b, and Gag/Δp2b-ESCRT fusions with HA-tagged full-length avian Nedd4 (HA-LDI-1 FL) in 293E cells, immunoprecipitated the Gag complexes from the lysate fraction with an anti-HA serum, and fractionated the proteins by SDS-PAGE. ASV Gag was detected by Western blotting using anti-ASV polyclonal antiserum (Fig. 6, upper panel), and Nedd4 was detected using an anti-HA-antiserum (lower panel). Nedd4 co-immunoprecipitated with wild type Gag but not Gag/Δp2b as expected (lanes 1 and 2, respectively). The co-immunoprecipitation of HA-Nedd4 and ASV Gag (lane 1) indicates that the HA tag did not prevent the binding of Nedd4 to Gag. When we examine the immunoprecipitates obtained with the Gag/Δp2b-ESCRT fusion proteins (lanes 3–9), Nedd4 did not co-immunoprecipitate, indicating that a stable complex between the Nedd4 E3 protein and Gag was lost when the PY motif was deleted in these constructs.

**Budding of ASV Gag Does Not Require Vps37C**—We previously demonstrated that fusion of Tsg101 (ESCRT-I) to Gag/Δp2b rescued VLP release but utilized a different mechanism than wild type ASV Gag (35). Because Vps37C (ESCRT-I) exhibits a significant degree of budding and ubiquitination when tethered to Gag/Δp2b, we asked whether there was an underlying role for Vps37C in ASV Gag budding. We co-transfected 293/E cells with the p203E vector encoding ASV Gag and a siRNA pool directed against Vps37C. As shown in Fig. 7, release of ASV VLPs remains mostly unaffected by transfection of siRNAs targeting Vps37C (lanes 2 and 4) or random sequence (lanes 1 and 3). In contrast, depletion of Vps37C by the specific siRNA (lanes 6 and 8) but not the nonspecific siRNA (lanes 5 and 7) blocked release of HIV-1 Gag. Exog-
enously expressed Vps37C was depleted in cells by greater than 95% by the specific (lane 10) but not the nonspecific (lane 9) siRNA under these conditions. These results suggest that Vps37C, similar to Tsg101, is not needed for release of ASV Gag. This is in contrast to HIV-1, which requires both of these proteins for the budding process.

Budding of HIV-1 Gag with a PTAP Point Mutation Is Rescued by Fusion to an ESCRT-III Protein—Because budding from cells by ASV Gag containing an L domain deletion was partially rescued by fusion to ESCRT-II and -III proteins, we asked whether HIV-1 Gag containing an L domain inactivating mutation, P7L, would also be rescued by fusion to the same ESCRT-III and -III proteins (Fig. 8). HIV-1 Gag-GFP and Gag-GFP containing the P7L mutation were expressed in cells, and Gag was detected in the lysate and media fractions by Western blotting as described under “Experimental Procedures.” As expected (9), Gag-GFP, which releases VLPs at levels similar to wild type HIV-1 Gag (2, 36, 37), was observed in the medium fraction, whereas release of Gag-GFP P7L VLPs into the medium fraction was significantly reduced (Fig. 8, compare lanes 1 and 2). In contrast to ASV Gag/Δp2b, Eap20 failed to complement the L domain mutation in HIV Gag/P7L (Fig. 8, lane 3). The Gag/P7L-Chmp6 chimera exhibited a high budding efficiency (Fig. 8, lane 5). We repeated this experiment using HIV-1 Gag constructs without the GFP and labeled cells with [35S]-Met/Cys. Consistent with the results of the Western analysis shown in Fig. 8, Gag/P7L-Chmp6 rescued budding to ~80% wild type level whereas fusion to Eap20 did not rescue budding. The only difference in the results shown in Fig. 8 and the labeling experiment was that we detected some rescue by the Gag/P7L-Chmp4B not detected by immunoblotting (data not shown). Taken together, the finding that ESCRT proteins differ in ability to complement the L domain functions of ASV and HIV-1 Gag suggests that determinants outside of the L domain modulate budding events or that there are multiple and distinct pathways being used for virus release.

FIGURE 8. ESCRT proteins covalently linked to HIV-1 Gag rescue the budding defect caused by the P7L mutation. The cells were transfected with plasmids encoding HIV-1 Gag-GFP, Gag/P7L-GFP, or C-terminal fusions of various ESCRT proteins to HIV Gag/P7L as indicated. VLPs were harvested from the media and lysate fractions as described in the legend to Fig. 2, except that an anti-HIV CA (p24) monoclonal antibody was used to immunoprecipitate Gag from the cell lysate. The proteins were detected by Western blotting with a monoclonal antiserum directed at HIV-1 CA (p24). The top panel shows cellular expression levels of HIV-1 Gag-GFP (lane 1), Gag/P7L-GFP (lane 2), and Gag/P7L fused to Eap20 (lane 3), to Eap45 (lane 4), to Chmp6 (lane 5), and to Chmp4B (lane 6). The bottom panel shows VLPs released into the media. Note: HIV Gag/P7L-Eap45 was co-expressed with Eap20-FLAG in this experiment.

FIGURE 9. Fragments of Chmp6 cause dominant negative interference of ASV and HIV-1 Gag budding from cells. The effect of full-length and C-terminally deleted (1–167) Chmp6 on ASV Gag (top panel) or HIV-1 Gag-GFP (middle panel) release. VLPs were harvested from media fractions of 293/E cells transfected with HIV Gag-GFP or ASV Gag and indicated Chmp6 constructs, including full-length Chmp6-FLAG (Chmp6 FL) and Myc-Chmp6 1–167. Cell lysates (left panel) and VLPs (right panel) were subjected to Western blot analysis using anti-ASV mAb MA(p19) or anti-GFP monoclonal antibodies. Chmp6 constructs in cell lysates were visualized by immunoblotting with an anti-FLAG or anti-Myc antibody. WT, wild type.

A Fragment of Chmp6 Is a Potent Dominant Negative Inhibitor of Both ASV and HIV-1 Gag Release from 293E Cells—Because Chmp6 complemented the L domain mutations of ASV and HIV-1 Gag, although with very different efficiencies, we next determined whether there was a common requirement for this ESCRT-III protein. We first used a siRNA depletion approach. Although we could demonstrate depletion of an exogenously expressed Chmp6-FLAG protein by >90% with a specific siRNA, release of ASV and HIV-1 Gag appeared unaffected (data not shown). HIV-1 Gag release was previously reported to be unaffected by this protocol (23). We therefore used a dominant negative interference approach. Recent structure/function analyses by Shim et al. (22) predict that the ESCRT-III proteins contain six α helices and that deletion of the most C-terminal helix alters normal protein function by inducing homopolymerization on cellular membranes. To ascertain whether Chmp6 is required for ASV Gag budding, we co-transfected 293/E cells with an expression plasmid encoding ASV Gag along with one encoding Chmp6 with a deletion that removes the most C-terminal α helix. As a control, we show in Fig. 9 that expression of the full-length Chmp6-FLAG (residues 1–201) had no effect on the release of HIV-1 (lane 11) or ASV Gag (lane 5). On the other hand, overexpression of Myc-Chmp6(1–167) results in a potent inhibition of HIV Gag-GFP release (lane 12) in a dominant negative manner, as shown previously (22). Similarly, expressing Myc-Chmp6(1–167) inhibits the release of ASV Gag (lane 6), suggesting that both HIV-1 and ASV Gag share a common requirement for Chmp6 and its ESCRT-III function. We observe a lower expression level of Myc-Chmp6-(1–167) in the cytosolic fraction relative to Chmp6-FL (lanes 14 and 15).

siRNA Depletion of Eap20 Blocks ASV but Not HIV-1 Gag Release—The observation that translational fusion of Eap20 did not efficiently rescue the HIV-1 Gag P7L mutation is consistent with the report that depletion of the endogenous protein from cells has little detectable effect on HIV-1 Gag release (9). We
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A. Eap20 siRNA

Mock 20 nM

Eap20-FLAG

Fig. 10. Depletion of Eap20 from cells blocks ASV but not HIV-1 Gag release. FLAG-tagged Eap20 was co-transfected into cells with an Eap20-specific siRNA or random siRNA negative control. Proteins recovered from the lysate fraction were resolved by SDS-PAGE, and the expression level of FLAG-tagged Eap20 was detected with an antiserum directed at the FLAG tag in the presence or absence of 20 nm siRNA specific for Eap20 (upper panel, lanes 1 and 2) or 20 nm random control (lower panel, lanes 1 and 2). Mock represents cells transfected with the Eap20-FLAG expression vector but not the siRNA targeting Eap20. Eap20 suppression because transfection of a random, nontargeting siRNA control had no effect on depletion of Eap20.

B. Lysate and VLPs

WT ASV Gag

Lysate: Mock Eap20

VLPs: Mock Eap20

HIV Gag-GFP

siRNA: Mock Eap20

WT ASV Gag

siCONTROL

Mock siCONT

Mock siCONT

Mock siCONT

These results indicate that ASV but not HIV-1 Gag utilizes Eap20 for release from cells.

DISCUSSION

In our studies, we aimed to functionally substitute the ASV L domain with full-length components of the ESCRT sorting machinery believed to function in budding. The most efficient rescue of VLP release occurred by transfectionally linking Gag/Δp2b to the ESCRT-I complex using Tsg101 or Vps37C. This suggests that ESCRT-I most closely substituted for the required Nedd4 functions, but by a mechanism more similar to HIV-1 Gag release. Additionally, Langelier et al. (23) previously reported that HIV-1 does not utilize ESCRT-II for release. The presence and functional use of an AIP1-binding site in the p6 and NC regions of HIV-1 Gag suggests that AIP1 may act as a bridging factor to link HIV-1 Gag to ESCRT-III. Deletion of a putative AIP1-binding motif, LYP3L, found in ASV Gag suggests that the ESCRT-II activity to link the budding complex to downstream components of the ESCRT pathway. Consistent with this interpretation, we found that depletion of Eap20, in cells using an siRNA suppression strategy blocked the release of ASV but not HIV-1 Gag. In contrast to their effect on HIV Gag P7L, the ESCRT-III component Chmp6, only marginally rescued budding of chimeric ASV Gag/Δp2b. The efficient rescue of the HIV-1 Gag P7L by fusion to Chmp6 suggests that different components of the ESCRT-III complex may be utilized by ASV and HIV-1. However, both ASV and HIV-1 Gag release were subject to potent dominant negative interference by overexpression of a fragment of Chmp6 containing amino acids 1–167 of the protein.

Rescue of ASV VLP release by the ESCRT-I and -II complexes restored ubiquitin modification of chimeric Gag/Δp2b in the lysate fraction; monoubiquitinated forms of the ESCRT chimeras were detected in VLPs. This suggests that ubiquitin plays a signaling role in budding, perhaps serving as a ligand for assembly of the ESCRT complexes early in the budding process. Stuchell et al. (26) also detected higher molecular mass species corresponding to monoubiquitinated forms of HIV-1 Gag-Vps37B. The inability of the nonrescuing ESCRT chimeras to restore ubiquitination may account for their poor budding efficiency. Additionally, ubiquitination of chimeric Gag/Δp2b constructs entails that these complexes must interact with E3 ubiquitin ligases in a productive budding pathway. The finding that the Nedd4-E3 protein does not co-immunoprecipitate with Gag/Δp2b-ESCRT fusions suggests that a new E3 protein may be recruited to the complex, possibly by one of the ESCRT proteins, to restore the ubiquitination.

Although the ASV Gag/Δp2b-Chmp6 chimera exhibits some budding, it did not acquire detectable levels of ubiquitin modification in the lysate or VLPs. This suggests that ubiquitin signaling functions in earlier stages of the MVB pathway corresponding to ESCRT complexes with ubiquitin-binding domains. Consistent with this idea is the proposed model that ESCRT-I and -II complexes function as adaptors to recognize and sort ubiquitinated cargo, and ESCRT-III functions primarily in cargo concentration and vesicle formation (10). Alternatively, the inability to detect ubiquitinated forms of the ESCRT-III chimeras may indicate the association of deubiquitinating enzymes, such as associated molecule of SH3 domain of STAM (AMSH), with the ESCRT-III complex (39, 40). Several studies therefore asked whether depletion of Eap20 had an inhibitory effect on ASV Gag release (Fig. 10). Under conditions where levels of FLAG-tagged EAP20 were significantly depleted by transfection of an Eap20-specific siRNA (Fig. 10A), release of HIV-1 Gag-GFP transfection into the cells was unaffected (Fig. 10B, lane 8) as previously reported (23). In contrast, release of ASV Gag transfected into cells with Eap20-specific siRNA was inhibited by ~87% relative to the siRNA control-treated cells (Fig. 10B, lane 4). This inhibition was specific for Eap20 suppression because transfection of a random, nontargeting siRNA control had no effect on depletion of Eap20-FLAG (Fig. 10A, lower panel) or release of ASV Gag (Fig. 10B, lane 12). These results indicate that ASV but not HIV-1 Gag utilizes Eap20 for release from cells.

5 Dolan and J. Leis, unpublished observation.
report that deubiquitination of endosomal cargo precedes MVB biogenesis. Additionally, Agromayor and Martin-Serrano (41) showed that catalytically inactive AMSH inhibited murine leukemia virus Gag budding, although siRNA-mediated deplet- 

on of AMSH did not affect release. From our data, we observed a reduction in the amount of ubiquiti- nated Gag in VLPs compared with Gag in the lysate fraction, suggesting that the ubiquitin moieties were removed or excluded from Gag during budding.

Tethering Hrs or ESCRT-I proteins to the C terminus of HIV-1 Gag complement the budding defect caused by an L domain mutation (21, 24–26). In this report, we demonstrate that Chmp6 (ESCRT-III) but not Eap20 (ESCRT-II) rescues HIV-1 Gag from the inhibitory effects of an L domain-inactivating mutation. This is almost opposite of what is observed with ASV Gag/Δp2b, where Eap20 is more effective than Chmp6 in the rescued. There is now accumulating evidence that although ASV and HIV-1 share some common elements of a budding pathway under the control of the Vps4 ATPase, they proceed through different protein complexes to bud from cells. This evidence includes the facts that: (a) dominant negative interference by 3’ fragments of Tsg101 block HIV-1 but not ASV Gag release from 293E cells (14); (b) siRNA-mediated depletion of endogenous cellular levels of Tsg101 blocks HIV-1 release from human (293E) cells (13) but not ASV Gag release from avian (DF-1) cells (35); (c) as described here, siRNA depletion of endogenous cellular levels of Vps37C blocks HIV-1 but not ASV Gag release, whereas depletion of Eap20 blocks ASV but not HIV-1 Gag release; (d) L-domain-inactivating mutations in ASV and HIV-1 Gag, respectively, are differentially rescued by fusion to ESCRT-II and -III proteins; and (e) VLPs assembled from ASV Gag-GFP and Gag/Δp2b -Eap20 are labeled with N-(rhodamine)-phosphatidylethanolamine, an endocytic membrane marker, whereas VLPs assembled from HIV-1 Gag, ASV Gag/Δp2b-Tsg101, or Gag/Δp2b-Vps37C are not (35). This last observation suggests that wild type ASV Gag and Gag/Δp2b-Eap20 bud or pass through a different set of membranes than HIV-1 Gag, ASV Gag/Δp2b-Tsg101, or ASV Gag/Δp2b-Vps37C chimeras. Taken together, these results support the conclusion that ASV and HIV-1 Gag utilize different subsets of ESCRT proteins to facilitate the budding process using parallel egress pathways.

We previously showed that translational fusion of ubiquitin to the C terminus of ASV Gag failed to rescue particle release inhibited by dominant negative Nedd4 mutants, suggesting that Nedd4-like proteins provide additional functions during budding besides Gag modification (18). C-terminal fusion of ubiquitin rescues ASV and HIV-1 Gag budding defects caused by chemical depletion of free ubiquitin in cells, indicating that these constructs are biologically functional (42, 43). Apparently, fusing ESCRT factors to the L-domain mutant bypassed this requirement. Possibly, Nedd4-like proteins function as adaptors to link ASV Gag to ESCRT components of the MVB pathway. This notion is supported by the observation that several Nedd4-like proteins accumulate on aberrant endosomes, called class E compartments, induced by dominant negative forms of Vps4 (44). Also, Tsg101 interacts with Nedd4 in cells (16). Alternatively, the C2 transport domain of Nedd4 may be required for Gag transport to a specific region of the plasma membrane in conjunction with the M domain signal associated with the N-terminal half of the mAb protein, and as noted above and described elsewhere (35), the fused ESCRT factor effectively compensated for this targeting.

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