Identification of Human VPS37C, a Component of Endosomal Sorting Complex Required for Transport-I Important for Viral Budding*

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Endosomal sorting complex required for transport-I (ESCRT-I) is one of three defined protein complexes in the class E vacuolar protein sorting (VPS) pathway required for the sorting of ubiquitinated transmembrane proteins into internal vesicles of multivesicular bodies. In yeast, ESCRT-I is composed of three proteins, VSP23, VPS28, and VPS7, whereas in mammals only Tsg101(VPS23) and VPS28 were originally identified as ESCRT-I components. Using yeast two-hybrid screens, we identified one of a family of human proteins (VPS37C) as a Tsg101-binding protein. VPS37C can form a ternary complex with Tsg101 and VPS28 by binding to a domain situated toward the carboxyl terminus of Tsg101 and binds to another class E VPS factor, namely Hrs. In addition, VPS37C is recruited to aberrant endosomes induced by overexpression of Tsg101, Hrs, or dominant negative form of the class E VPS ATPase, VPS4. Enveloped viruses that encode PTAP motifs to facilitate budding exploit ESCRT-I as an interface with the class E VPS pathway, and accordingly, VPS37C is recruited to the plasma membrane along with Tsg101 by human immunodeficiency virus, type 1 (HIV-1) Gag. Moreover, direct fusion of VPS37C to HIV-1 Gag obviates the requirement for a PTAP motif to induce virion release. Depletion of VPS37C from cells does not inhibit murine leukemia virus budding, which is not mediated by ESCRT-I, however, if murine leukemia virus budding is engineered to be ESCRT-I-dependent, then it is inhibited by VPS37C depletion, and this inhibition is accentuated if VPS37B is simultaneously depleted. Thus, this study identifies VPS37C as a functional component of mammalian ESCRT-I.

The multivesicular body (MVB) serves as an important organelle for the sorting of endocytosed and newly synthesized transmembrane proteins (1–3). A major decision governing the fate of such proteins is made when they either remain on the limiting membrane of the MVB or become internalized within the lumen of the MVB. Specifically, proteins that are sorted into luminal vesicles of the MVB are ultimately targeted to the lumen of the lysosome/vacuole for degradation (1–3). A number of protein factors that comprise the “class E” vacuolar protein sorting (VPS) machinery function at the MVB limiting membrane to select membrane proteins for luminal targeting and to induce the formation and budding of vesicles into the MVB lumen. Most of these class E VPS factors are components of protein complexes, and at least three “endosomal sorting complexes required for transport” (ESCRT-I, -II, or -III) (4–6) are linked by an elaborate series of protein-protein interactions between their components and/or bridging factors (7–11). Cargo sorting and vesicle formation likely involves sequential recruitment of ESCRT-I, -II, and -III, and subsequent vesicle fission requires dissociation of ESCRT complexes from the MVB membrane, induced by an AAA-ATPase, VPS4 (12, 13).

The propensity of the class E VPS factors to induce membrane fission and vesicle release is exploited by enveloped viruses. Many encode sequence motifs, termed late assembly or “L” domains to recruit class E VPS factors to mediate budding of nascent virions through cellular membranes. Three classes of L domains have been described and are defined by the presence of essential P(T/S)AP, YPXXLF, or PPXY peptide motifs (18–20). PTAP motifs occur in the L domains of, for example, HIV-1 and Ebola virus (18, 21), and recruit (ESCRT-I) via a direct interaction with the ESCRT-I component Tsg101 (22–25). PTAP binds to the amino-terminal ubiquitin E2 variant-like (UEV) domain, and this interaction serves to recruit Tsg101 to sites of virus particle assembly at the plasma membrane (24, 26, 27). However, the UEV domain is apparently dispensable if the remaining portion of the protein is artificially recruited to virus assembly sites by tethering to an HIV-1 Gag protein (24). A second component of ESCRT-I, VPS28, binds to a carboxyl-terminal domain of Tsg101 and is recruited along with it to HIV-1 particle assembly sites (27, 28). Moreover, the VPS28-binding activity of Tsg101 is required in order for it to support HIV-1 budding (27, 29) suggesting that VPS28 also plays an important role in the budding of HIV-1 and probably other enveloped viruses that encode a PTAP-type L domain. Enveloped viruses that exploit alternative L domain classes.
apparently do not require ESCRT-I but do require at least some of the remaining class E VPS proteins during their egress (7–9, 23, 27).

In mammals, Tsg101 and VPS28 were, until recently, the only identified components of ESCRT-I. However, the orthologous yeast complex contains an additional component, VPS37 (4), that does not have obvious sequence homologues in mammals. Therefore, we set out to identify mammalian orthologues of VPS37 by yeast two-hybrid screening. This search yielded hVPS37C, one of a family of proteins, which includes VPS37A and VPS37B, that was also recently identified as ESCRT-I components (29, 30). Here, we describe the characterization of VPS37C, its interactions with Tsg101 and other class E VPS factors and show that it, along with VPS37B, plays a crucial role in mediating the virus release activity of PTAP-type viral L domains.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmids based on pCR3.1/YFP that express human class E VPS factors fused to the carboxy terminus of YFP have been previously described (7, 27). pCR3.1/YFP derivatives were constructed and are identical to the pCR3.1/YFP derivatives, except for the color of the expressed fluorescent fusion protein. The VPS37C and VPS3B open reading frames (see “Results” for description of the VPS37C open reading frame) were similarly inserted into pCR3.1/YFP for expression as YFP fusion proteins in mammalian cells. pCR3.1-based plasmids expressing a synthetic, codon-optimized HIV-1 Gag cDNA, and a derivative lacking p6 have been previously described (27). A plasmid expressing an HIV-1 Gag-HcRed fusion protein was constructed by inserting the respective codon optimized cDNAs into the mammalian expression vector pCAGGS. A plasmid expressing a GST-VPS37C fusion protein in mammalian cells was constructed by inserting the VPS37C coding sequence into pCAGGS/GST, pGBK77 and pVP16-derived plasmids that express the GAL4 DNA binding domain and yeast activator domain fusion proteins, respectively, have either been described previously (7, 27) or were constructed by inserting PCR-amplified coding sequences of VPS37C and VPS37B into pVP16.

Yeast Two-hybrid-based Screening and Assays—A Y190-derived yeast culture carrying pGBK77/Tsg101 was generated by standard transformation and selection procedures. These yeast were transformed with a library of cDNAs derived from peripheral mononuclear lymphocytes (PML39) in the pGAD10 vector (Clontech). Approximately 2 million transformants (selected by growth on media lacking tryptophan and histidine) were screened by an X-gal (5-bromo-4-chloro-3-indolyl-

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transformants (selected by growth on media lacking tryptophan and histidine) with a library of cDNAs derived from peripheral mononuclear lymphocytes (PML39) in the pGAD10 vector (Clontech). Approximately 2 million transformants (selected by growth on media lacking tryptophan and histidine) were screened by an X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) assay (23). After confirmation of hits, the clone was subcloned into yeast plasmids expressing a synthetic, codon-optimized HIV-1 Gag cDNA, and a derivative lacking p6 have been previously described (27). A plasmid expressing an HIV-1 Gag-HcRed fusion protein was constructed by inserting the respective codon optimized cDNAs into the mammalian expression vector pCAGGS. A plasmid expressing a GST-VPS37C fusion protein in mammalian cells was constructed by inserting the VPS37C coding sequence into pCAGGS/GST, pGBK77 and pVP16-derived plasmids that express the GAL4 DNA binding domain and yeast activator domain fusion proteins, respectively, have either been described previously (7, 27) or were constructed by inserting PCR-amplified coding sequences of VPS37C and VPS37B into pVP16.

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GST and YFP Fusion Protein Coprecipitation Assay—293T cells in 12-well plates were cotransfected with 250 ng of pCR3.1/YFP-VPS37C or pCR3.1/YFP-VPS37B and 250 ng of pCR3.1/CFP-Tsg101, pCR3.1/CFP-VPS28, pCR3.1/CFP-Hrs, pCR3.1/CFP-VPS4, or pCR3.1/CFP-VPS4(DN). In some experiments, 750 ng of pCR3.1/HIV-1 Gag, pCR3.1/HIV-1 GagΔp6, or pCAGGS/Gag–HcRed was cotransfected with pCR3.1/YFP-VPS37C and pCR3.1/CFP-Tsg101. Twenty-four hours post-transfection cells were fixed in 3% paraformaldehyde for 25 min, washed with 10 mM glycine in phosphate-buffered saline, and visualized. Fluorescence images were acquired using an Olympus IX70 inverted microscope-based DeltaVision system (Applied Precision, Issaquah, WA). Optical filters were used to distinguish CFP (436/10 nm excitation, 500/40 nm emission), YFP (520/40 nm emission), and HcRed (575/25 nm excitation, 630/40 nm emission). Respective excitation wavelengths were separated from fluorescence emission using a triple-band dichroic beam-splitter (Omega Optical #XP2090, Brattleboro, VT).

RESULTS

Yeast Two-hybrid Screening and Identification of VPS37C—We screened a peripheral mononuclear lymphocyte library using a GAL4-Tsg101 fusion protein as a bait and obtained 77 hits. After plasmid rescue and retransformation, three of these clones tested positive for interaction with GAL4-Tsg101 but not with GAL4 alone. Sequence analysis of one of these clones, initially termed PML39, revealed that it encoded a protein of 355 amino acids, and this clone was selected for further study. The predicted open reading frame (lacking 5′- and 3′-noncoding sequences present in the originally isolated clone) was subcloned so as to express a VP16 activation domain fusion protein, and this protein also tested positive in the yeast two-hybrid assay in combination with a GAL4-Tsg101 bait (Fig. 1A).

The protein predicted to be encoded by the PML39 clone contained a Mod(r) (modifier of rudimentary) domain (33) at its amino terminus, and BLAST searches of the human sequence databases revealed that the protein was identical to the carboxy-terminal 355 residues of a 529-amino acid hypothetical protein, termed PLJ20847. In addition, the predicted amino-terminal 160 amino acids of PML39 had significant homology to another hypothetical human protein, FLJ1270, as well as to hepatocellular carcinoma-related protein 1 (HCRP1) and Williams Beuren syndrome chromosome region 24 (WBSCR24). Interestingly, PML39 also shared a similar degree of homology with a Drosophila protein (CG1115-PA), which has been shown to bind to Drosophila Tsg101 during the course of a genome wide screen of Drosophila protein-protein interactions (34).

An alignment of the amino-terminal 159 amino acids of PML39, which encodes the Mod(r) domain, with these homologues is shown in Fig. 1B. While this report was in preparation, human HCRP1 and FLJ1270 were independently identified by yeast two-hybrid screening and homology searches as potential mam-
malian orthologues of yeast VPS37 (29, 30). These two proteins were designated VPS37A and VPS37B, and, based on sequence homology, FLJ20847 (hypothetical) and WBSCR24 were termed VPS37C and VPS37D. Although FLJ20847/VPS37C was not characterized further in these studies, one study showed that a Mod(r) domain-containing fragment of FLJ20847/VPS37C binds to Tsg101 in vitro (30).

The hypothetical 523-amino acid FLJ20847/VPS37C protein sequence, whose carboxy terminus aligned perfectly the 355-amino acid PML39 coding sequence that we isolated, was deposited in the Refseq data base during the course of this work. However, the FLJ20847 hypothetical protein extends the amino terminus of VPS37C by 168 additional amino acids beyond that of the PML39 clone. Nonetheless, several lines of evidence suggested that the PML39 clone encoded the full-length VPS37C protein. First, a more recent BLAST search of human expressed sequence tags with sequences encoding the amino terminus of the hypothetical FLJ20847 protein from Drosophila, dmVPS37 (CG1115PA).

**VPS37C Binding Sites in Tsg101**—We next determined which domains of Tsg101 were responsible for its VPS37C-binding activity. Yeast two-hybrid analyses, shown in Fig. 2A, showed that a Tsg101 UEV domain (29). VPS37C, on the other hand, does not contain any PTAP motifs and did not bind to the Tsg101 UEV domain (29). VPS37C bound to more than one peptide determinant in Tsg101, because non-overlapping Tsg101 fragments (1–303 and 303–390) each had VPS3C-binding activity. We also tested whether VPS37B could bind to components of ESCRT-I, namely Tsg101 and VPS28, when expressed in mammalian cells. Glutathione-Sepharose-based precipitation analysis using a GST-VPS37C fusion protein expressed in 293T cells showed that a coexpressed YFP-Tsg101 fusion protein, but not YFP alone, could be very efficiently coprecipitated with GST-VPS37C (Fig. 2A). In contrast, a YFP-VPS28 fusion protein was not efficiently coprecipitated with GST-VPS37C, and only trace amounts of VPS28 were detected in the glutathione-bound fraction (Fig. 2B). However, when YFP-Tsg101 and YFP-VPS28 were coexpressed, both were efficiently coprecipitated with GST-VPS37C (Fig. 2B, lanes 2). When a mutant YFP-Tsg101 fusion protein, that contains a four amino acid to alanine substitution in residues 368–371 and does not bind well to VPS328 (previously termed mutant A3 (27)), was coexpressed with GST-VPS37C and YFP-VPS28, the precipitable complex contained YFP-Tsg101(A3) but not YFP-VPS28 (Fig. 2B, lanes 3). Thus, the ability of GST-VPS37C to associate with YFP-VPS28 was dependent on Tsg101 and we therefore conclude that the three proteins can form a ternary complex, with VSP28 and VPS37C each possessing the ability to bind to Tsg101, but not to each other. This conclusion is also consistent with findings obtained using yeast two-hybrid assays, where Tsg101 bound to both VPS37C and to VPS28 (Fig. 1A), but VPS37C and VPS28 did not bind to each other (see Fig. 4 below).

**VPS37C Forms a Ternary Complex with Tsg101 and VPS28 in Human Cells**—We next determined whether VPS37C could bind to components of ESCRT-I, namely Tsg101 and VPS28, when expressed in mammalian cells. Glutathione-Sepharose-based precipitation analysis using a GST-VPS37C fusion protein expressed in 293T cells showed that a coexpressed YFP-Tsg101 fusion protein, but not YFP alone, could be very efficiently coprecipitated with GST-VPS37C (Fig. 2A). In contrast, a YFP-VPS28 fusion protein was not efficiently coprecipitated with GST-VPS37C, and only trace amounts of VPS28 were detected in the glutathione-bound fraction (Fig. 2B). However, when YFP-Tsg101 and YFP-VPS28 were coexpressed, both were efficiently coprecipitated with GST-VPS37C (Fig. 2B, lanes 2). When a mutant YFP-Tsg101 fusion protein, that contains a four amino acid to alanine substitution in residues 368–371 and does not bind well to VPS328 (previously termed mutant A3 (27)), was coexpressed with GST-VPS37C and YFP-VPS28, the precipitable complex contained YFP-Tsg101(A3) but not YFP-VPS28 (Fig. 2B, lanes 3). Thus, the ability of GST-VPS37C to associate with YFP-VPS28 was dependent on Tsg101 and we therefore conclude that the three proteins can form a ternary complex, with VSP28 and VPS37C each possessing the ability to bind to Tsg101, but not to each other. This conclusion is also consistent with findings obtained using yeast two-hybrid assays, where Tsg101 bound to both VPS37C and to VPS28 (Fig. 1A), but VPS37C and VPS28 did not bind to each other (see Fig. 4 below).

**VPS37C Binding Sites in Tsg101**—We next determined which domains of Tsg101 were responsible for its VPS37C-binding activity. Yeast two-hybrid analyses, shown in Fig. 2B, revealed that VP16-VPS37C bound to GAL4-fused carboxy-terminal Tsg101 fragments containing residues 157–390, 250–390, and 303–390. A similar carboxy-terminal deletion analysis revealed that VPS37C bound to Tsg101 fragments containing residues 1–157. Therefore, VPS37C bound to more than one peptide determinant in Tsg101, because non-overlapping Tsg101 fragments (1–303 and 303–390) each had VPS3C-binding activity. We also tested whether VPS37B could bind to GAL4-fused Tsg101 fragments. In this case we found that VSP37B bound to all Tsg101 fragments. This is probably because VPS37B contains a PTAP motif that binds to the amino-terminal Tsg101 UEV domain (29). VPS37C, on the other hand, does not contain any PTAP motifs and did not bind to the Tsg101 UEV domain (Fig. 2B).
A complimentary coprecipitation analysis done by coexpressing GST-VPS37C and various Tsg101 fragments fused to YFP in 293T cells was consistent with the yeast two-hybrid assay results. These experiments, shown in Fig. 2C, demonstrated that YFP-fused Tsg101 fragments containing residues 1–360, 1–303, 157–390, 250–390, or 303–390 coprecipitated with GST-VPS37C, whereas those containing residues 1–157, 1–250, or 330–390 did not. Thus we conclude that there are multiple contact sites between Tsg101 and VPS37C and that Tsg101 residues 250–303 and 303–330 contain the major determinants that independently contribute to the overall Tsg101-VPS37C interaction. However, we are not able to determine whether there exists one large contiguous binding site or several separate contact sites at the interface(s) between Tsg101 and VPS37C.

Colocalization of VPS37C and VPS37B with Tsg101-induced Vesicles—Fluorescent microscopic examination of cells expressing YFP-VPS37C (with unfused CFP as a tracer) revealed that the YFP-VPS37C fusion protein accumulated in puncta, whereas unfused CFP was distributed diffusely throughout the cell (Fig. 3A). We did not characterize these YFP-VPS37C accumulations further, and it was not clear whether these represented vesicle structures or artifactual protein aggregates. However, upon coexpression with CFP-Tsg101, YFP-VPS37C assumed a very different localization and accumulated in vesicular structures (Fig. 3A) that we have previously shown to be formed by overexpressed fluorescent protein-Tsg101 fusions (27). Indeed, when coexpressed together, CFP-Tsg101 and YFP-VPS37C fluorescence signals were nearly perfectly coincident (Fig. 3A). In contrast, when YFP-VPS37C was coexpressed with CFP-VPS28, each protein localized largely independently (Fig. 3A), with YFP-VPS37C present in discrete puncta and with CFP-VPS28 predominantly assuming its normal, diffuse distribution, as previously reported (27). We did, however, observe a marginal increase in CFP-VPS28 signal intensity coincident with YFP-VPS37C puncta. These microscopic analyses are consistent with the notion that VPS37C and VPS28 associate with Tsg101 independently and do not bind directly to each other. We also found that YFP-VPS37B localized in puncta when overexpressed alone and, surprisingly, these were in the nucleus (Fig. 3B). However, upon coexpression with CFP-Tsg101, YFP-VPS37B, like VPS37C, colocalized almost perfectly with CFP-Tsg101 in cytoplasmic vesicular structures.

Interaction and Colocalization of VPS37C with Other Class E VPS Factors—Given that VPS37C appeared to be a bona fide Tsg101-binding protein, we next used yeast two-hybrid assays to test whether a VPS37C further participated in the complex network of protein-protein interactions that comprise the mammalian class E VPS pathway (7, 9). Specifically we tested whether a GAL4-VPS37C fusion protein could bind to any known components of mammalian ESCRT-II and ESCRT-III as well as factors that act upstream of ESCRT-I (Hrs) and factors that bridge the ESCRT-I and ESCRT-III complexes (AIP1/ALIX). As is shown in Fig. 4A, these results were generally negative, except that VPS37C bound to Hrs and weakly to AIP-1/ALIX, each of which are themselves Tsg101-binding proteins (7–9, 14–16). The VPS37C-Hrs interaction was readily confirmed using the GST fusion protein coprecipitation assay with plasmids expressing VP16-VPS37C or VP16-VPS37B. The resulting levels of β-galactosidase expression in optical density units is shown. C, coprecipitation analysis of VPS37C binding to Tsg101 fragments. GST-VPS37C was coexpressed in 293T cells with unfused YFP or YFP-fused to the indicated Tsg101 residues. Both unfractionated cell lysates and glutathione-bound fractions (as indicated) were subjected to Western blot analyses using anti-GFP antibody.

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** VPS37C forms a ternary complex with Tsg101 and VSP28 by binding to a carboxyl-terminal domain of Tsg101. A, coprecipitation of Tsg101 and VPS28 with VPS37C. GST-VPS37C was coexpressed in 293T cells with unfused YFP or YFP-Tsg101 (left panels), and both unfractionated cell lysates as well as glutathione bound fractions (as indicated) were subjected to Western blot analysis using an anti-GFP antibody. Alternatively (right panels), a similar analysis was done, except that combinations of YFP-VPS28 and wild-type or VPS28 non-binding mutant forms of YFP-Tsg101 were coexpressed with GST-VPS37C. The numbers to the left of the blots indicate the migration positions of molecular weight markers, and the positions of YFP-Tsg101, YFP-VPS28, and unfused YFP are shown to the right. B, yeast two-hybrid analysis of VPS37C and VPS37B binding to Tsg101 fragments. A schematic representation of the various Tsg101 domains is shown, and yeast were cotransformed with plasmids expressing the indicated GAL4 DNA binding domain fused Tsg101 fragments along with plasmids expressing VP16-VPS37C or VP16-VPS37B. The resulting levels of β-galactosidase expression in optical density units is shown. C, coprecipitation analysis of VPS37C binding to Tsg101 fragments. GST-VPS37C was coexpressed in 293T cells with unfused YFP or YFP-fused to the indicated Tsg101 residues. Both unfractionated cell lysates and glutathione-bound fractions (as indicated) were subjected to Western blot analysis using an anti-GFP antibody.
Tsg101. A Hrs interaction appears robust and easily demonstrable (Fig. 4, with VP16-VPS37C) failed to detect any interaction between orientation of the yeast two-hybrid assay (GAL4-AIP1/ALIX by coprecipitation (data not shown). Moreover, the reciprocal AIP1/ALIX in the yeast two-hybrid assay could not be detected each other. The interaction observed between VPS37C and Stuchell was not recruited to CFP-Hrs-induced vesicles, even though YFP-VPS37C under conditions of CFP-Hrs overexpression and C

(Fig. 4B), and overexpression of CFP-Hrs and YFP-VPS37C resulted in their nearly perfect colocalization in large cytoplasmic vesicles that were induced by CFP-Hrs overexpression (Fig. 4C). Interestingly, YFP-VPS37B behaved differently to YFP-VPS37C under conditions of CFP-Hrs overexpression and was not recruited to CFP-Hrs-induced vesicles, even though Stuchell et al. (29) reported that these two proteins can bind to each other. The interaction observed between VPS37C and AIP1/ALIX in the yeast two-hybrid assay could not be detected by coprecipitation (data not shown). Moreover, the reciprocal orientation of the yeast two-hybrid assay (GAL4-AIP1/ALIX with VP16-VPS37C) failed to detect any interaction between the two proteins (data not shown). Thus, although the VPS37C-Hrs interaction appears robust and easily demonstrable (Fig. 4, A–C), whether VPS37C and AIP1/ALIX bind directly to each other remains somewhat uncertain. Hrs binds to another protein, HBP/STAM, which, for technical reasons, we are unable to test in the yeast two-hybrid assay (35). However, we were able to coprecipitate YFP-HBP/STAM using GST-VPS37C (Fig. 4B). Whether this interaction is direct or is bridged by endogenous Hrs is unclear at present. Nonetheless, these experiments indicate the existence of a relatively complex series of protein interactions involving VPS37C, Tsg101, VPS28, Hrs, HBP/STAM, and AIP-1/ALIX.

Many of the proteins that comprise the class E VPS pathway do not bind directly to the class E VPS ATPase, VPS4 (7, 9, 10), and this appeared true for VPS37C, based on findings in the yeast two-hybrid assays (Fig. 4A) and in GST fusion protein coprecipitation assays (Fig. 4B). Nonetheless, a characteristic property of class E VPS factors is that they generally accumulate on aberrant endosomes induced by VPS4 ablation in yeast or by expression of catalytically inactive, dominant negative (DN) VPS4 mutants in mammalian cells (5, 6, 12, 13, 28). This is likely because most class E VPS factors, including VPS4 itself, cycle on and off the limiting membrane of late endosomes, and their disassembly and release require VPS4 activity (12). VPS4(DN)-induced compartments can be visualized directly by expressing fluorescent fusion proteins fused to VPS4(DN). As can be seen in Fig. 4D, overexpression of a wild type form of CFP-VPS4, which is distributed diffusely in the cytoplasm at steady state, did not alter the cytoplasmic punctate distribution of YFP-VSP37C. However, upon coexpression with CFP-VPS4(DN), YFP-VPS37C accumulated on aberrant CFP-VPS4(DN)-induced endosomes. Thus, in addition to its ability to bind and colocalize with Tsg101 and Hrs, VPS37C displays additional properties of a bona fide class E VPS factor.

Relocalization of Tsg101-VPS37C Complexes to the Plasma Membrane by HIV-1 Gag—Previously, we have shown that Tsg101 can be relocalized to sites of viral budding at the plasma membrane by HIV-1 Gag or Ebola virus matrix proteins (24, 27), both of which encode PTAP-type L domains. Moreover, VPS28 can also be recruited by these viral proteins, provided that it is in a complex with Tsg101 (27). To test whether Tsg101-VPS37C complexes could be recruited by viral proteins, we coexpressed CFP-Tsg101, YFP-VPS37C, and HIV-1 Gag-HcRed fusion proteins. As can be seen in Fig. 5A, under these conditions a substantial fraction of the YFP-VPS37C protein localized to the plasma membrane along with CFP-Tsg101 and HIV-Gag-HcRed. A minor fraction of HIV-1 Gag-HcRed, CFP-Tsg101, and YFP-VPS37C colocalized on intracellular vesicles. In the absence of CFP-Tsg101, YFP-VPS37C was not recruited by HIV-1 Gag (data not shown). As a specificity control we also compared the ability of unlabeled full-length HIV-1 Gag with Gag lacking the p6 domain (GagΔp6) to induce VPS37C relocalization to the plasma membrane. As can be seen in Fig. 5B, recruitment of CFP-Tsg101-YFP-VPS37C complexes was dependent on the ability of Gag to bind Tsg101, because, unlike full-length Gag, GagΔp6 failed to induce CFP-Tsg101-YFP-VPS37C relocalization. Because we have previously shown that VPS28 is also recruited to viral budding sites in a p6- and Tsg101-dependent manner (27), we conclude from this analysis that it is likely that viral proteins that make use of a PTAP-type L domain likely recruit the entire ESCRT-I complex.

Tethering VPS37C to the Site of HIV-1 Budding Rescues a Late Budding Defect Induced by PTAP Mutation—Previously, we showed that artificial tethering of Tsg101 to sites of HIV-1 particle assembly could suppress a budding defect induced by mutation of the PTAP L domain (24, 27). The experimental approach used to demonstrate this was based on complemen-
tation of a budding defective (PTAP mutant) HIV-1 whose defect in release could be rescued by coexpression by a Gag protein in which p6 was deleted and Tsg101 was instead fused at its carboxyl terminus. We adopted the same approach using VPS37C. As can be seen in Fig. 6, controls showed that a PTAP-defective HIV-1 provirus can quite efficiently generate virion particles when complemented in trans by a Gag protein containing an intact p6 domain, or Tsg101 in place of p6. Conversely, undetectable levels of virions are released when the complementing Gag protein contains p6 bearing a PTAP mutation. Importantly, a Gag protein containing VPS37C in place of p6 efficiently complemented PTAP mutant virion release. We also confirmed the recent finding of Stuchell et al. (29) that VPS37B also has this activity. Overall, these experiments show that tethering VPS37C (or Tsg101 or VPS37B) to sites of HIV-1 particle assembly can obviate the requirement for a PTAP motif in HIV-1 budding, consistent with the notion that VPS37C is a component of ESCRT-I and can, at least if it is appropriately recruited, act as an interface with the class E VPS pathway.

Depletion of VPS37B and VPS37C Specifically Inhibits PTAP-ESCRT-I-dependent Viral Budding—Finally, we determined whether VPS37C was required for PTAP-dependent viral budding. For these analyses we used a previous described approach employing recombinant murine leukemia viruses (MLVs) in which the endogenous PPPY-type L domain is either left intact, or is replaced with a peptide sequence derived from HIV-1 that includes the PTAP-type L domain (MLV/p6) (32). The advantage of this approach is that the wild type MLV serves as an excellent control: whereas MLV budding requires

![Image](http://www.jbc.org/Downloadedfrom)
some components of the class E VPS pathway (7, 23), it does not apparently require ESCRT-I and is largely unaffected by Tsg101 depletion using siRNA, or most dominant-negative Tsg101 fragments (except those that cause general perturbation of the class E VPS pathway) (23, 27, 36). Thus, if VPS37C is a genuine component of ESCRT-I, any effects of its perturbation on viral budding should, in principle, be specific to MLV/p6 while leaving wild-type MLV unaffected.

As can be seen in Fig. 7A, siRNA duplexes directed against Tsg101, VPS37B, and VPS37C effectively silenced expression of the respective YFP fusion proteins upon cotransfection. Therefore, we next tested whether the siRNAs inhibited viral budding and found that wild type MLV release was quite insensitive to Tsg101, VPS37B, or VPS37C siRNA (Fig. 7B, left panels). In contrast, MLV/p6 release was attenuated by Tsg101 siRNAs, as expected, and was at least partly inhibited by VPS37B or VPS37C siRNA. The most dramatic effects were observed when both VPS37B and VPS37C siRNAs were transfected, and under these conditions, very few mature MLV/p6 virions were released. Wild type MLV release and maturation remained, however, only marginally affected by simultaneous VPS37B/VPS37C siRNA transfection. Importantly, neither Tsg101 nor VPS37B/VPS37C siRNAs deleteriously affected the level of MLV Gag expression in cells. For reasons that are unclear, MLV/p6 release as measured by pelletable p30CA levels in culture supernatants appeared more sensitive to ESCRT-I component depletion than did that measured by Pr65 Gag precursor release. We suspect that this is because retardation of MLV release may perturb the ordered series of proteolytic cleavage events that accompany virus maturation that is initiated during budding. Importantly, similar effects are observed when MLV L domain function is attenuated by mutation rather than cofactor depletion (data not shown). Nonetheless, these findings document a clear and specific effect of VPS37B/VPS37C depletion on PTAP-dependent virion release.

FIG. 5. Recruitment of VPS37C to the plasma membrane by HIV-1 Gag. A, YFP-VPS37C was coexpressed with CFP-Tsg101 and Gag-HcRed. Images were acquired and analyzed by deconvolution microscopy. Images acquired with the CFP filter set (upper left) are pseudo-colored blue, and are combined with those acquired with the YFP filter set (upper right, pseudo-colored green) and the HcRed filter set (lower left, pseudo-colored red), to generate overlaid images (lower right). B, YFP-VPS37C was coexpressed with CFP-Tsg101 and unlabeled wild-type HIV-1 Gag (top row) or HIV-1 Gag lacking the p6 domain (bottom row).

FIG. 6. Tethering VPS37C to sites of HIV-1 particle assembly obviates the requirement for a PTAP motif. Western blot analysis, using an anti-CA antibody of cell lysates and extracellular virions (as indicated) generated following transfection with a PTAP-mutant HIV-1 proviral plasmid and plasmids expressing an HIV-1 Gag protein in which p6 was intact (p6), or carried mutations in the PTAP motif (PTAP mut). Alternatively p6 was replaced with Tsg101, VPS37C, or VPS37B, as indicated.

DISCUSSION

In yeast, ESCRT-I is composed of a ternary complex of Vps23, Vps28, and Vps37 (4), whereas in mammals only Tsg101 and Vps28 had been identified as ESCRT-I components at the outset of this study. We identified a Tsg101-binding protein, Vps37C, that fulfills many of the criteria for the “missing” mammalian orthologue of yeast Vps37. Several homologues of Vps37C exist in humans, and while this manuscript was in preparation this family of proteins was identified using homology searching with yeast Vps37 and two members, Vps37A and Vps37B, were also identified by yeast two-hybrid screening (29, 30). Thus, it appears that the single Vps37 protein in yeast has been elaborated during mammalian evolution such that humans encode at least four orthologues. Interestingly, similar elaboration has occurred among other components of the class E VPS machinery, for instance the six yeast Chmp proteins have been elaborated to ten in humans, indicating that the class E machinery, while essentially conserved, may work in a slightly more complex manner or has evolved to deal with a more complex array of substrates in mammals than in yeast.

The protein whose identification is described here and is termed Vps37C, binds to a carboxyl-terminal domain of Tsg101 and forms a ternary complex with Tsg101 and Vps28.
anti-GFP antibody. Directed against luciferase (Luc) as a control, or Tsg101, VSP37B, or YFP-VPS37B, or YFP-VPS37C along with the indicated siRNA pools.

293T cells were cotransfected with plasmids expressing YFP-Tsg101, APTAP-dependent MLV budding.

This property is shared with VPS37A and VPS37B (29, 30). A deletion analysis revealed that at least two Tsg101 determinants participate in its interaction with VPS37C. Tsg101-VPS37B interactions appear even more extensive, because every Tsg101 fragment tested bound to VPS37B to some extent. As has been recently shown, this is likely due to the presence of a PTAP motif in VPS37B that mediates weak interactions with the amino-terminal Tsg101 UEV domain (29). We were unable to detect any interaction between VPS37C and VPS28 in the absence of overexpressed Tsg101, suggesting that Tsg101 constitutes the core “bridging” component of ESCRT-I.

Interestingly, there are some apparent differences among the various VPS37 forms in terms of their ability to bind other proteins that could potentially be functionally important. For example, in contrast to VPS37C (this study) and VPS37B (29), it appears that VPS37A is able to bind to VPS28 (30). All three VPS37 forms tested can bind to Hrs, but there does appear to be some difference in the efficiency with which this occurs, because YFP-VPS37C colocalized almost perfectly with CFP-Hrs-induced vesicles upon coexpression in mammalian cells, whereas YFP-VPS37B showed little or no tendency to colocalize with CFP-Hrs. Both VPS37B and VPS37C proteins did colocalize with vesicles induced by CFP-Tsg101 overexpression or by dominant-negative mutants of CFP-VPS4 (Ref. 29 and this study), consistent with the notion that both are bona fide class E VPS factors. Another interaction that we observed in the yeast two-hybrid assay was between VPS37C and AIP1/ALIX. Unfortunately, this could not be confirmed using coprecipitation techniques; thus its significance is unclear. Nonetheless, YFP-VPS37C and CFP-AIP1/ALIX colocalized when expressed together in mammalian cells (data not shown).

Although it is certainly possible that ESCRT-I complexes are quite homogeneous and stable, and contain relatively invariant proportions of Tsg101, VPS28, and VPS37A, -B, -C, and -D, the slightly different properties of each VPS37 protein hint at potentially heterogeneous forms and functions for ESCRT-I complexes. It is intriguing that neither Tsg101/VPS32 nor VPS28 has been elaborated during mammalian evolution, yet there appear to be four distinct forms of VPS37. Thus, heterogeneity in ESCRT-I function, if it occurs, should be specified by VPS37.

At present, the precise composition of ESCRT-I in mammals remains to be determined, but both Tsg101 and VPS37 are capable of multimerization (Refs. 27 and 29, and data not shown) as well as binding to each other and VPS28. Moreover, the presence of UEV-binding PTAP motifs in Hrs, Tsg101, and VPS37B, all of which are also interconnected by PTAP-independent interactions, and at least three of which can homomultimerize (7, 9, 14–16), is suggestive of the existence of a potentially very broad array of similar, but compositionally and conformationally distinct, protein complexes. Whether the associations among ESCRT-I components and their binding partners are dynamic and regulated during protein trafficking is a potentially interesting question that remains to be addressed.

Many enveloped viruses exploit the class E VPS machinery to bud from cells, and there appear to be, in principle, several alternative routes by which the pathway can be accessed by viruses. For HIV-1 and EIAV that normally use Tsg101 and/or AIP1/ALIX as L domain cofactors, the L domain can be rendered superfluous by artificially tethering ESCRT-I components to sites of virion assembly. Indeed, fusion of fragments of Tsg101 or Hrs to an HIV-1 Gag protein (16, 24, 29), or VPS28 to an HIV-1 or EIAV Gag protein (37), can rescue a budding defect induced by mutation of the Gag-encoded L domain motif. Here, we show that artificially tethering VPS37C to sites of HIV-1 assembly, by fusion to Gag, can efficiently suppress a viral budding defect induced by mutation of the viral PTAP motif. Stuchell et al. (29) recently reported a similar result using VPS37B. These data strongly argue that these two forms of VPS37 are genuine components of the ESCRT-I complex and can be used to recruit the class E VPS pathway to mediate viral budding.

Additionally, we show that VPS37B and VPS37C depletion inhibits the budding of an MLV strain that is engineered to be PTAP/ESCRT-I dependent, while not significantly affecting wild-type MLV budding that is dependent on components of the class E VPS pathway other than ESCRT-I (23). Interestingly, depletion of either VPS37B or VPS37C individually clearly reduced MLV/p6 budding, but depletion of both had even more dramatic effects. This suggests that both Tsg101-VPS37B and Tsg101-VPS37C complexes can participate in PTAP-dependent viral budding. Whether VPS37B and VPS37C serve to recruit additional factors required for budding or modulate Tsg101 conformation or stability is not known at present. VPS37A

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depletion did not apparently affect Tsg101 stability (30), but it is possible that Tsg101 could be alternatively stabilized by any one of the four VPS37 forms. Nonetheless, depletion of two of the four VPS37 species had clear effects on PTAP-dependent viral budding, indicating that the VSP37B and VPS37C-containing forms of ESCRT-I are important for viral budding activity and almost certainly for at least a subset of cellular cargo sorting and vesicular budding events at MVBs.

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