The Human Endosomal Sorting Complex Required for Transport (ESCRT-I) and Its Role in HIV-1 Budding*[S]◆

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Efficient human immunodeficiency virus type 1 (HIV-1) budding requires an interaction between the PTAP late domain in the viral p6[Gag] protein and the cellular protein TSG101. In yeast, Vps23p/TSG101 binds both Vps28p and Vps37p to form the soluble ESCRT-I complex, which functions in sorting ubiquitylated protein cargoes into multivesicular bodies. Human cells also contain ESCRT-I, but the VPS37 component(s) have not been identified. Bioinformatics and yeast two-hybrid screening methods were therefore used to identify four novel human proteins (VPS37A–D) that share weak but significant sequence similarity with yeast Vps37p and to demonstrate that VPS37A and VPS37B bind TSG101. Detailed studies produced four lines of evidence that human VPS37B is a Vps37p ortholog. 1) TSG101 bound to several different sites on VPS37B, including a putative coiled-coil region and a PTAP motif. 2) TSG101 and VPS28 co-immunoprecipitated with VPS37B-FLAG, and the three proteins comigrated together in soluble complexes of the correct size for human ESCRT-I (~350 kDa). 3) Like TGS101, VPS37B became trapped on aberrant endosomal compartments in the presence of VPS4A proteins lacking ATPase activity. 4) Finally, VPS37B could recruit TSG101/ESCRT-I activity and thereby rescue the budding of both mutant Gag particles and HIV-1 viruses lacking native late domains. Further studies of ESCRT-I revealed that TSG101 mutations that inhibited PTAP or VPS28 binding blocked HIV-1 budding. Taken together, these experiments define new components of the human ESCRT-I complex and characterize several TSG101 protein/protein interactions required for HIV-1 budding and infectivity.

Like other enveloped viruses, HIV-1[1] must bud from producer cells to spread infection. HIV budding requires both cis-acting viral elements and trans-acting cellular proteins (reviewed in Ref. 1). Efficient release and replication of HIV-1 in most cell types, including primary cells, require a conserved cis-acting P(S/T)AP motif (the "late domain"), located in the C-terminal p6 region of the structural Gag protein (2–5). The P(S/T)AP late domain binds and recruits the cellular protein TSG101 (tumor susceptibility gene 101), which facilitates efficient separation of the viral and cellular membranes during the final stages of virus release (6–9). In addition to HIV-1, several other pathogenic human viruses, including human T-cell leukemia virus type I (Retroviridae), Ebola virus (Filoviridae) and Lassa virus (Arenaviridae), also use P(S/T)AP late domains to recruit TSG101 during virus budding (7, 10–13). In turn, TSG101 helps to recruit a complex set of cellular machinery that is normally used for protein sorting, membrane distortion, and fission at the multivesicular body (MVB) (1).

Much of our understanding of MVB biogenesis comes from studies in yeast (reviewed in Refs. 14 and 15). Vps23p, the yeast ortholog of TSG101, binds two other proteins, Vps28p and Vps37p, to form a stable soluble ~350-kDa complex called ESCRT-I (endosomal sorting complex required for transport) (16). ESCRT-I is recruited to the endosomal membrane through an interaction with the upstream Vps27p-HRS complex (17–21). Once on the membrane, ESCRT-I helps recruit a series of downstream proteins and complexes (the "Class E" proteins), which collectively sort ubiquitylated proteins into vesicles that bud into the maturing endosome to create MVBs. In most cases, these protein sorting and vesiculation events ultimately target proteins for degradation in the interior of the lysosome (vacuole in yeast).

Although the human MVB pathway is more complex and less well characterized than the yeast pathway, there is now good evidence for conservation of ESCRT-I composition and function from yeast to humans. First, TSG101 and VPS28 are required for the ubiquitin-dependent delivery of cathepsin D and endocytosed receptors to the lysosome (e.g. Refs. 17 and 22–24). Second, mammalian TSG101 and VPS28 also function together as part of a soluble ~350-kDa ESCRT-I complex (22, 25). However, a significant limitation in our understanding of the human ESCRT-I complex is that orthologs of VPS37 have not yet been identified in animals.

As illustrated in Fig. 1A, TSG101 is a multifunctional, multidomain protein composed of an N-terminal ubiquitin E2 variant (UEV) domain (residues 1–145), a proline-rich region (res-

idues ~146–215), a predicted central coiled-coil region (residues ~240–311), and a conserved helical C-terminal domain (residues ~330–390). TSG101 appears to function primarily as an adaptor protein, and the different regions of TSG101 engage in a series of protein/protein interactions within the ESCRT-I complex, including the following. 1) The TSG101 N-terminal UEV domain binds P(S/T)AP peptide motifs. Potential cellular binding sites include a downstream PTAP motif within TSG101 itself and P(S/T)AP motifs within at least two other Class E proteins, HRS and AIP1 (17–20, 26–28). 2) The C-terminal region of TSG101/Vps23p binds VPS28/Vps28p (14, 25, 29). This interaction is important for MVB formation and may also control the steady-state levels of TSG101 (30). 3) Finally, deletion of a predicted central coiled-coil region within Vps23p blocks incorporation of Vps37p into the ESCRT-I complex, suggesting that this region of Vps23p may form (or contain) the Vps37p-binding site (16).

Given the parallels between MVB vesicle formation and HIV-1 budding, it has become important to characterize the different TSG101 protein/protein interactions that function in ESCRT-I assembly and test their involvement in virus budding. The goals of this study were therefore to identify human VPS37 orthologs and to test the importance of known TSG101/ESCRT-I interactions in HIV-1 budding.

MATERIALS AND METHODS

VPS37 Homology Searches—In one procedure, the yeast Srn2p/Vps37p protein sequence (NCBI accession number NP_013220) was submitted to the NCBI PSI-BLAST search engine and compared with all known proteins. The first iteration identified apparent VPS37 orthologs in fungi and Japanese rice (NCBI accession number AAS01952). A composite profile built from these sequences was then compared with all known proteins to identify VPS37 orthologs in animals. All sequences above the default threshold (18 total) were incorporated into the search profile, and a final iteration identified four human proteins: VPS37A/HCRP1 (hepatocellular carcinoma-related protein 1; NCBI LocusLink 137492), VPS37B (NCBI LocusLink 79720), VPS37C (NCBI LocusLink 55048), and VPS37D/WBSCR24 (Williams-Beuren syndrome chromosome region 24; NCBI LocusLink 155382).

In a second procedure, the five proteins shown to bind TSG101 in a global analysis of protein/protein interactions in *Drosophila* (31) were compared against the human protein data base. One of the *Drosophila* proteins (CG1115-PA; NCBI accession number NP_649518) showed clear sequence similarities to VPS37B and VPS37C ($E \leq 1 \times 10^{-6}$) and weaker similarities to VPS37A ($E = 1.4$) and VPS37D ($E = 0.034$).

Plasmid Construction—Mammalian expression vectors for wild-type (WT) and PTAP mutant HIV-1$_{NL4-3}$, WT and mutant VPS4A, and small interfering RNA (siRNA)-resistant TSG101 (TSG*-FLAG) have been described (6). Additional point mutations were introduced into pIRES-TSG*-FLAG using Kunkel or QuikChange (Stratagene) mutagenesis. Deletion mutants were created by PCR amplifying the desired region of pIRES-TSG*-FLAG and subcloning the product into pIRES2-EGFP. VPS37B constructs were PCR-amplified from an expressed sequence tag (DDBJ/GenBank™/EBI accession number BE909752; American Type Culture Collection, Manassas, VA) with the sequence encoding the C-terminal FLAG epitope within the 3′-primer. VPS28 (GenBank™ accession number BC019321) constructs were PCR-amplified from a QUICK-Clone human HeLa cDNA pool (Clontech) using gene-specific primers. For expression in *Escherichia coli*, the VPS28 coding region was subcloned into WISP01-69, a modified pGEX2T vector (Amersham Biosciences), to create WISP01-136, which expresses VPS28 as a GST fusion protein.

Yeast Two-hybrid Binding Assays—Global and directed yeast two-hybrid screens were performed as described previously (19, 26). Briefly, directed two-hybrid assays with full-length TSG101 and VPS37B and deletion constructs were performed using the Matchmaker Gal4 yeast two-hybrid 3 system (Clontech). In each experiment, five test clones and five controls were selected at random and tested for α-galactosidase activity in −Leu/−Trp/−Ade/−His X-α-gal (50 µg/ml) plate assays. Directed two-hybrid assays with full-length TSG101 and VPS28 and deletion constructs were performed using the MP30 vector system (32). To identify yeast colonies producing β-galactosidase, cells were lifted onto filter paper disks, lysed by freeze-thawing in liquid nitrogen, and assayed with X-β-gal. For quantitation of β-galactosidase activity, ~20 colonies/plate were randomly selected, pooled, grown on synthetic liquid medium, and assayed with chlorophenol red β-D-galactopyranoside or *o*-nitrophenyl-β-D-galactopyranoside (6).

VPS37B-FLAG Immunoprecipitation Experiments—293T cells (10-cm plate) were transfected using LipofectAMINE 2000 (Invitrogen) with 13 µg of pCIneo-VPS37B-FLAG or the control pCIneo-FLAG vector. Cells were harvested after 24 h; lysed for 30 min on ice in 1 ml of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Nonidet P-40 containing 2 µM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 5 µg/ml aprotinin; and then clarified by centrifugation at 16,000 × *g* for 20 min. Lysate (~1 mg of total protein) was incubated overnight at 4 °C with anti-FLAG antibody M2-agarose affinity beads (Sigma, A 2220). Unbound proteins were removed by lysis buffer washes, and bound proteins were stripped by boiling in SDS-PAGE buffer and analyzed by Western blotting.

Western Blotting—Virus from the supernatants of transfected cells was pelleted through a 20% sucrose cushion in a microcentrifuge at 13,000 rpm for 90 min at 4 °C and resuspended in 25 µl of 1× SDS gel loading buffer. Samples (5–7.5 µl) were separated by 12% SDS-PAGE, transferred, blocked (typically with 5% nonfat dry milk, but as low as 0.1% when detecting endogenous TSG101), blotted with antisera, and protein bands were detected by enhanced chemiluminescence (ECL, Pierce). Transfected human embryonic kidney 293T cells (1 well from a 6-well plate) were harvested directly into 40–45 µl of radioimmune precipitation assay buffer (10 mM Tris-Cl (pH 7.0), 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS) and incubated on ice for 4 min. Samples were clarified by microcentrifugation at 13,000 rpm for 4 min at 4 °C, and lysates were resuspended in an equal volume of 2× SDS gel loading buffer. Aliquots (5–10 µl) were resolved by 12–17.5% SDS-PAGE. Bound antibody was detected by ECL using SuperSignal West Pico (Pierce) according to the manufacturer's instructions. The following primary antibodies were used: rabbit anti-HIV CA protein antibody (1:2000), rabbit anti-HIV MA protein antibody (1:25,000; Didier Trono, Geneva, Switzerland), murine monoclonal anti-TSG101 antibody 4A10 (1:100–1000; GeneTex, Inc.), murine monoclonal anti-FLAG antibody M2 (1:5000; Sigma), and rabbit anti-NEDD4 antibody (1:5000; Pharmingen). Anti-VPS28 antibodies (1:500–1500) were raised against recombinant VPS28 (see below) in New Zealand White rabbits (Covance, Inc.).

Gel Filtration—293T cells transfected with pCIneo-VPS37B-FLAG (as described for the immunoprecipitation experiments) were harvested after 24 h; lysed for 30 min on ice in 1 ml of phosphate-buffered saline (pH 7.4) and 0.1% Tween 20 containing 2 µM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 5 µg/ml aprotinin; and clarified by centrifugation at 16,000 × *g* for 30 min. The lysate was fractionated by gel filtration chromatography on a Sephacryl S300 column (Amersham Biosciences). Proteins in the 1.4-ml fractions were trichloroacetic acid-precipitated, washed with acetone (22), and analyzed by Western blotting. Apparent molecular masses were obtained by comparison with a standard curve derived from chromatography of proteins of known mass.

Immunofluorescence—COS-7 cells grown on glass coverslips on 60-mm dishes were cotransfected with 100 ng of vector expressing VPS37B-FLAG and 900 ng of vector expressing WT or K173Q VPS4A-GFP using 2.5 µl of LipofectAMINE 2000 following the manufacturer's instructions. Cells were fixed and permeabilized 24 h post-transfection in 4% paraformaldehyde and phosphate-buffered saline, incubated with rabbit anti-FLAG antibody (1:500) at 4 °C for 16 h, washed with phosphate-buffered saline, and incubated with Alexa 594-conjugated goat anti-rabbit antibodies (1:500) to detect FLAG-tagged VPS37 proteins. Images were collected as single wavelengths on an Olympus FVX confocal fluorescent microscope using Fluoview Version 2.0.39 software. 0.3–0.7-µm-thick *Z* sections are shown.

Gag-VPS37B Rescue Experiments—All Gag and Gag-VPS37B expression constructs were based on pGag-GFP (a gift from Dr. Marilyn Resh) (33). Expression vectors for GagΔPTAP-GFP and GagΔp6 were created as described (19), and GagΔPTAP-VPS37B and Gag-VPS37B expression constructs were created by replacing the GFP coding region with the VPS37B coding sequence. Plasmid DNA encoding either Gag-GFP or Gag-VPS37B constructs was cotransfected together with GagΔp6 plasmid DNA into 293T cells (2 ml, 6-well plates). Protein expression and release of viruses and virus-like particles (VLPs) were analyzed by Western blotting 24 h post-transfection.

Viral Replication Assays—Infectivity of HIV-1 particles released into the supernatants from cells transfected with R9 constructs was determined in single-cycle MAGIC replication assays as described (34), except that infections were performed at three different dilutions in triplicate in 48- or 96-well plates. Blue cells and syncytia were counted 2 days post-infection.

TSG101 Depletion/Replacement Experiments—Synthetic 21-nucleotide siRNA duplexes with 2-nucleotide 2'-deoxythymidine 3'- or uridine 3'-overhangs were targeted to TSG101 coding nucleotides 413–433 (35). 293T cells in 6-well plates (2-ml culture volume) were transfected twice at 24-h intervals using LipofectAMINE 2000 (6). The first transfection was with 50 nM siRNA duplexes and either 2 μg of pSL1180 carrier plasmid (Amersham Biosciences) or 2 μg of WT or mutant siRNA-resistant pIRES-TSG*-FLAG expression vector. The second transfection was performed with the same siRNA duplex (50 nM) and 500 ng of vector R9. Viral supernatants and cytoplasmic lysates were harvested after an additional 24–28 h and analyzed for protein expression and virus infectivity. All transfections and subsequent analyses were repeated at least three independent times.

Recombinant VPS28 Purification—*E. coli* BL21(DE3) cells transformed with WISP01-136 were induced with 1 mM isopropyl-β-D-thiogalactopyranoside to express recombinant GST-VPS28 and grown for 4 h at room temperature. Following cell lysis, soluble GST-VPS28 was purified by glutathione-Sepharose affinity chromatography (GSTPrep FF, Amersham Biosciences) and eluted with 20 mM glutathione. Following dialysis in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM dithiothreitol, the fusion protein was cleaved with 1 mg of tobacco etch virus protease/100 mg of protein for 24 h at 23 °C; free GST was removed by two rounds of glutathione-Sepharose affinity chromatography; and VPS28 protein was purified to homogeneity by anion exchange chromatography on immobilized Q-Sepharose (Amersham Biosciences). VPS28 eluted at ~300 mM NaCl from a linear gradient of 50 mM to 1 M NaCl in 25 mM Tris-HCl (pH 8.0) and 5 mM 2-mercaptoethanol.

Detection of Proteins Incorporated into Virus Particles—Human MT-4 T-cells were infected with HIV-1$_{\rm NL4-3}$ by co-culture as described previously (36). 60 h post-infection, a supernatant was harvested, and virus was purified by pelleting through a 20% (w/w) sucrose cushion, followed by velocity centrifugation through an iodixanol gradient as described (37). Particles were concentrated by ultracentrifugation, and the purity of the samples was verified by SDS-PAGE, followed by silver staining. For Western blotting, virus particles corresponding to 1 μg of CA protein (adjusted by comparison with purified recombinant CA protein) (38) and 1 μg of recombinant CA protein (control) and extracts from 10^5 uninfected MT-4 cells were separated by 17.5% SDS-PAGE (200:1 acrylamide/bisacrylamide).

RESULTS

Identification of Candidate Human VPS37 Proteins—As discussed above, the human ESCRT-I complex presumably contains TSG101, VPS28, and VPS37. However, VPS37 orthologs have not yet been identified in humans or any other higher eukaryote, apparently because the primary sequence has diverged considerably from yeast Vps37p (which shares <20% sequence identity with any known human protein). We therefore pursued three different approaches to identify VPS37 candidates, and these approaches *independently* identified the same family of human proteins.

In the first approach, the yeast Vps37p protein sequence was used to search for human VPS37 candidates by creating PSI-BLAST composite profiles of putative VPS37 orthologs from higher eukaryotes (see "Materials and Methods" for details). Four human proteins were identified using this approach: VPS37A/HCRP1 (39), VPS37B, VPS37C, and VPS37D/WB-SCR24. In the second approach, we searched for human homologs of the five proteins previously shown to bind TSG101 in a global map of *Drosophila* protein/protein interactions (31). This approach again yielded the same four proteins as the best human protein matches to one of the five *Drosophila* TSG101-binding partners (Fig. 1*B*). Finally, we performed extensive yeast two-hybrid screens of human spleen cDNA libraries with the goal of identifying human TSG101-binding proteins *de novo*. These screens employed 12 different TSG101 fragments as baits and identified two different "minimal" fragments of VPS37A as binding partners for TSG101-(12–326) (identified nine times in total) (Table I) and one fragment of VPS37B (VPS37B-(1–126), identified once) as a binding partner for TSG101-(12–326).

VPS37A–D are most similar within an ~150-amino acid

region that has been designated the mod_r domain (Fig. 1*B*) (40). This is also the region of highest homology to yeast Vps37p and to the putative *Drosophila* VPS37 ortholog. Each mod_r domain contains at least one segment that is strongly predicted to form a coiled coil, as does the yeast Vps37p protein. Interestingly, our yeast two-hybrid screening studies indicated that the TSG101-binding sites on VPS37A and VPS37B span at least part of the mod_r domain. Thus, the screening and alignment analyses suggested that all four human VPS37 proteins might be Vps37p orthologs that bind TSG101 via their conserved mod_r domains. To test this hypothesis, we selected VPS37B for detailed studies because 1) its domain structure most closely resembles that of yeast Vps37p; 2) its mod_r domain has the highest identity to the *Drosophila* VPS37 protein (Fig. 1*B*) (data not shown); and 3) VPS37B mRNA is expressed in a wide variety of tissues (see NCBI UniGene Cluster Hs.77870 *Homo sapiens*), including macrophages and lymphocytes, which are the natural hosts for HIV-1 replication.

Yeast Two-hybrid Binding Studies—"Reverse" library screens were performed using six different VPS37B bait fragments, and these screens identified four different TSG101 fragments as binding partners for one of the baits (VPS37B-(50–170)) (Table I). Thus, unbiased library screens that employed *either* VPS37B or TSG101 baits successfully identified the reciprocal protein as a binding partner. A series of directed yeast two-hybrid experiments were then performed to confirm the interaction and to map the TSG101/VPS37B interaction sites. As shown in Fig. 2*A*, full-length TSG101-DBD and VPS37B-AD proteins interacted strongly, whereas empty vector controls showed no detectable background binding. Deletion mapping experiments revealed that the TSG101 interaction site is located within a central fragment of VPS37B that spans residues 65–112. This minimal binding site is also contained in all of the VPS37B fragments identified in the library screens (Table I). Deletion of an additional 11 residues from the C terminus (to residue 101), which removed even more of the second predicted coiled-coil sequence in VPS37B, blocked TSG101 binding, supporting the idea that the coiled coil is important for the TSG101 interaction.

Deletion analyses were also performed to map the binding site for VPS37B on TSG101 (Fig. 2, *A* and *B*). These analyses were complicated, however, because all three TSG101 fragments initially tested bound VPS37B to some extent. Binding was weakest for TSG101-(1–145), which showed a weak interaction with the full-length VPS37B protein (Fig. 2*A*, *lower panel*) and a moderate interaction with VPS37B-(65–198) (Fig. 2*B*). TSG101-(1–145) corresponds to the PTAP motif-binding UEV domain, and VPS37B contains a PTAP motif (residues 185–188). We therefore tested whether the PTAP site on TSG101 mediates the TSG101-(1–145)/VPS37B-(65–198) interaction using a point mutation (M95A) within the TSG101 UEV domain that blocks PTAP binding (41). This mutation eliminated the TSG101-(1–145)/VPS37B-(65–198) interaction (Fig. 2*B*, *lower panel*), indicating that the TSG101 UEV domain did indeed contact VPS37B-(65–198) through the PTAP motif-binding groove. Similarly, we found that the pure recombinant TSG101 UEV domain (residues 1–145) bound specifically to a nine-amino acid peptide spanning the VPS37B PTAP site as analyzed in biosensor binding experiments (ELA<u>PTAP</u>LPY; $K_D = 155$ μM) (data not shown). Thus, the N-terminal TSG101 UEV domain binds directly, but weakly, to the VPS37B PTAP motif. Importantly, however, the TSG101 M95A mutation did not appreciably inhibit the interaction between the full-length TSG101 and VPS37B proteins (Fig. 2*A*, *lower panel*). Hence, the VPS37B PTAP motif is not the primary binding site for TSG101. This situation is analogous to the TSG101/HRS inter-
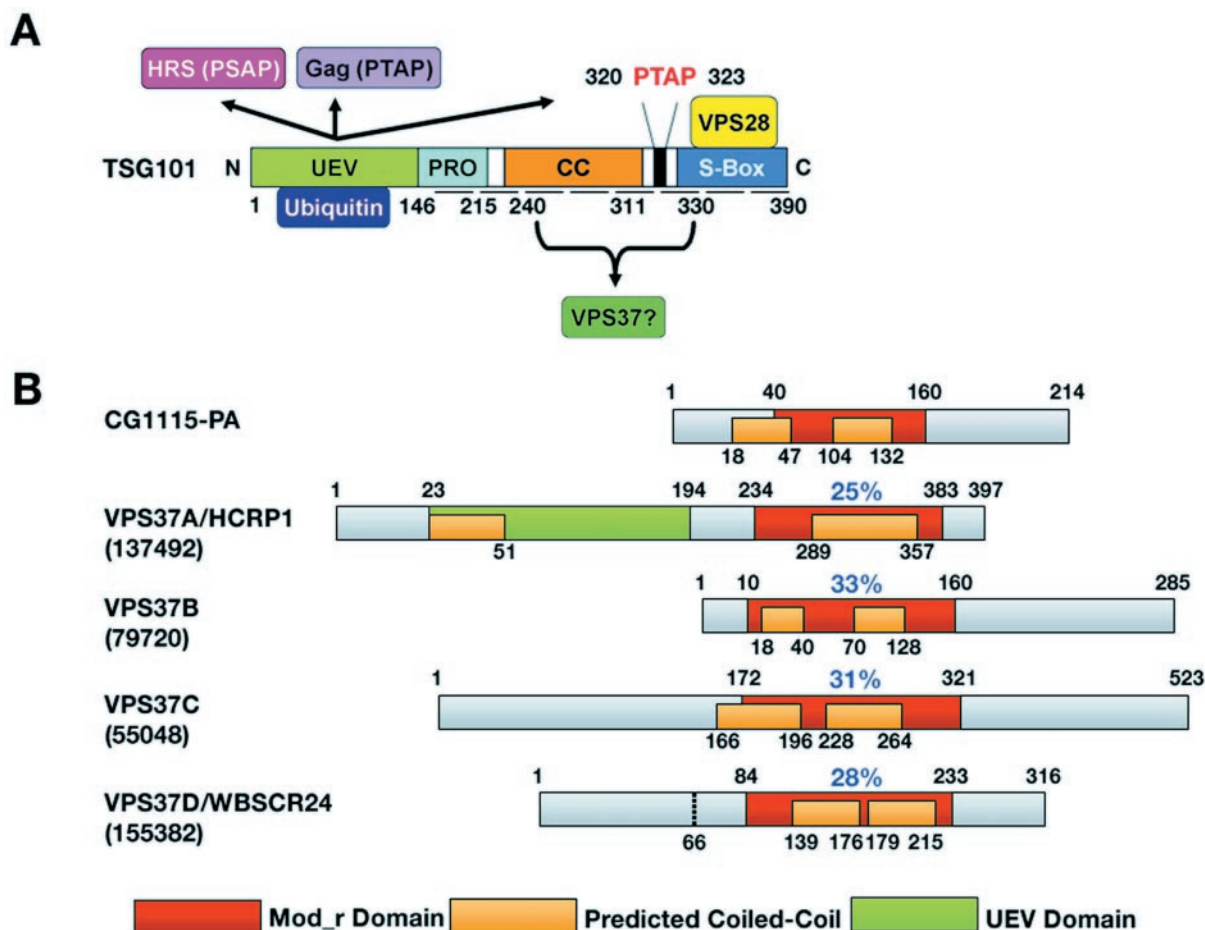
FIG. 1. **Schematic illustrations showing the putative domain structures and binding partners of TSG101 (*A*) and VPS37-like proteins (*B*).** *A*, schematic illustration of TSG101 showing the amino acid numbering scheme and approximate interaction sites of known binding partners. The *dashed line* highlights the observation that the C-terminal two-thirds of TSG101 can bind itself, although the precise sequence requirements for homo-oligomerization have not yet been determined (29). *PRO*, proline-rich region; *CC*, predicted coiled-coil region; *S-Box*, steadiness box region (30). Regions designated as coiled coils had a predicated coiled-coil propensity of >0.1 as analyzed by the program Multicoils (67) or >0.2 as analyzed by the program Coils (68). *B*, VPS37-like proteins from *Drosophila* (CG1115-PA) and humans (as indicated) are shown schematically, together with their NCBI LocusLink identifiers. Percent identities between the mod_r domains of CG1115-PA and the human proteins are shown in *blue*. Note that the N-terminal ends of the VPS37C and VPS37D/WBSCR24 proteins have not been determined experimentally, and the *dashed line* shows an alternative possible N terminus for VPS37D/WBSCR24. Putative domain identities were obtained from the Swiss Protein Knowledgebase (69), except that the E2 domain designation for VPS37A/HCRP1 was changed to "UEV" because the domain lacks an active-site cysteine residue.

TABLE I
TSG101/VPS37 interactions detected in yeast two-hybrid screens of human spleen libraries

Bait	Prey
TSG101-(12–326)	VPS37A-(194–397)
TSG101-(12–326)	VPS37A-(169–315)
TSG101-(12–326)	VPS37B-(1–126)
VPS37B-(50–170)	TSG101-(296–381)
VPS37B-(50–170)	TSG101-(297–390)
VPS37B-(50–170)	TSG101-(132–390)
VPS37B-(50–170)	TSG101-(208–335)

action, where the TSG101 UEV domain can bind an HRS PSAP sequence, but the primary interaction(s) between the two proteins occurs elsewhere (17–20).

TSG101 fragments spanning residues 158–309 and 310–390 also interacted with full-length VPS37B in the yeast two-hybrid experiments (Fig. 2*B*). Indeed, positive VPS37B interactions were detected for a series of non-overlapping fragments spanning the C-terminal end of TSG101 (Fig. 2*B*) (data not shown). It is therefore possible that VPS37B makes multiple contacts both upstream and downstream of TSG101 residue 310. However, we cannot rule out the alternative possibility that the observed TSG101/VPS37B interactions may, in some

cases, be bridged via the endogenous yeast ESCRT-I complex, particularly as determinants of TSG101 homo-oligomerization are also found in this region (29). In any event, these complications precluded a more precise mapping of the VPS37B-binding site on TSG101.

Finally, directed yeast two-hybrid experiments were also used to test for VPS37B interactions with other known human Class E proteins, and two human Class E proteins tested positive in these assays: VPS37B itself and HRS (data not shown). The former interaction indicates that VPS37B likely forms homo-oligomeric interactions. The latter interaction suggests that VPS37B may play an important role in helping the upstream HRS complex to recruit ESCRT-I to the endosomal membrane. We note that Bieniasz[2] has similarly found that the VPS37C protein interacts with HRS in two-hybrid assays. Yeast Vps37p exhibits two-hybrid interactions with three additional yeast Class E proteins (Vps20p, Vps28p, and Vps36p) (43), but we did not observe analogous interactions between the human orthologs in our experiments.

Human VPS37B Forms a Complex with TSG101 and VPS28 in Vivo—We next tested whether VPS37B forms stable com-

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[2] P. D. Bieniasz, personal communication.
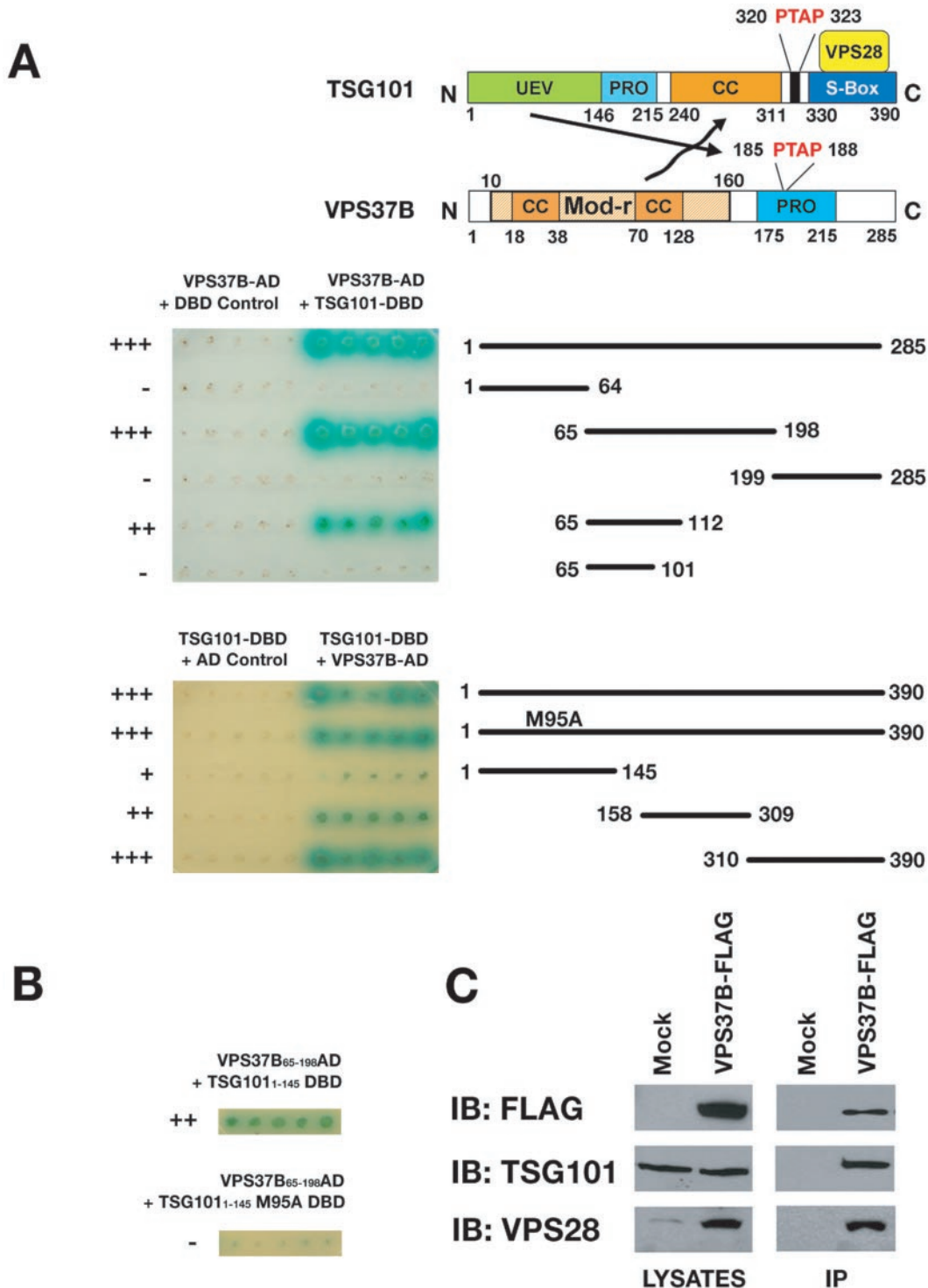
Fig. 2. **TSG101/VPS37B binding studies.** *A*, yeast two-hybrid binding signals arising from coexpression of the indicated pairs of test (*right*) or control (*left*) proteins. In the *upper panel*, a full-length TSG101-DBD fusion protein was coexpressed with the VPS37B-AD fusions shown to the right. In the *lower panel*, full-length VPS37B was coexpressed with the TSG101 fragments shown to the right. Note that VPS37B bound both TSG101-(1–145) and TSG101-(1–157) to a similar extent (not shown). Controls and test interactions were examined for five independent clones expressing the appropriate pairs of proteins. *Blue* indicates positive interactions, and the relative strength of each relevant interaction is summarized to the left. *PRO*, proline-rich region; *CC*, predicted coiled-coil region; *S-Box*, steadiness box region (30). *B*, yeast two-hybrid binding signals arising from coexpression of the designated pairs of proteins or controls. *C*, co-immunoprecipitation of endogenous TSG101 and VPS28 with VPS37B-FLAG. *Left panels*, Western blot signals observed for the input lysate (~50 μg of total protein input); *right panels*, Western blot signals following immunoprecipitation (*IP*) of VPS37B-FLAG (~500 μg of total protein input). *Right lanes*, lysates from cells expressing VPS37B-FLAG; *left lanes*, control cell extracts. *IB*, immunoblot.

plexes with TSG101 and VPS28 in human cells. As shown in Fig. 2*C* (*right panels*, *right lanes*), immunoprecipitation of exogenously expressed VPS37B-FLAG from soluble 293T cell extracts efficiently co-immunoprecipitated both TSG101 and

VPS28. This was a specific interaction, as neither TSG101 nor VPS28 was detected in control co-immunoprecipitation reactions with mock-transfected cells (*left lanes*). Interestingly, VPS37B overexpression also increased the steady-state levels

FIG. 3. **Gel filtration analyses of human ESCRT-I components.** Cells over-expressing VPS37B-FLAG protein were lysed, and the soluble lysate was fractionated by gel filtration chromatography. Fractions were analyzed by Western blotting to determine the elution profiles of TSG101, VPS28, and VPS37B. Western blots were digitized, and the intensities were plotted to show relative protein concentration. The elution positions of molecular mass standards are shown above the chromatograph and were used to estimate the apparent molecular masses of the different protein complexes (labeled above the peaks). *Inset*, Western blots from alternating fractions collected through the ESCRT-I elution profile. *IB*, immunoblot.
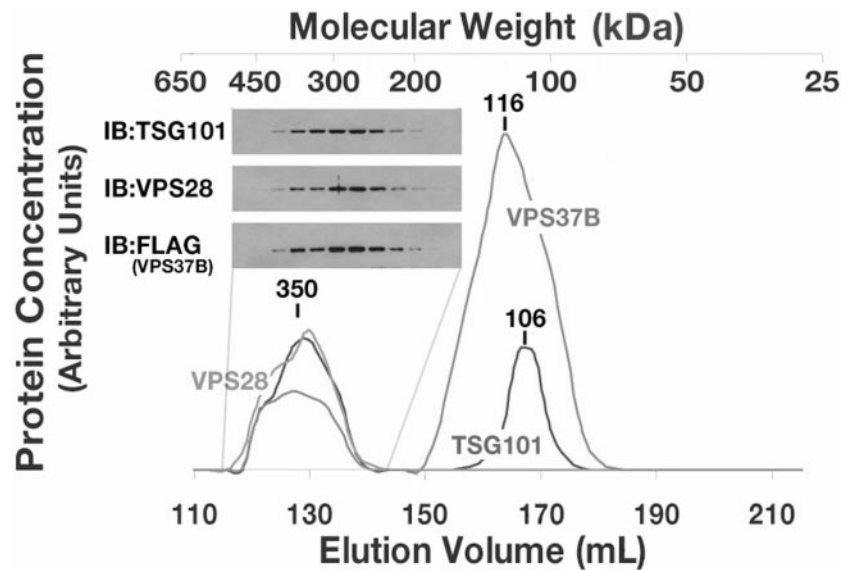

of VPS28 (*lower left panel*, compare the *two lanes*). This effect was observed in multiple experiments, but the relative increases in VPS28 levels varied between the different experiments. Thus, our data demonstrate that the TSG101, VPS28, and VPS37B-FLAG proteins associate within cells and indicate that elevated VPS37B levels can stabilize VPS28, presumably via ESCRT-I complex formation.

The Human ESCRT-I Complex—Gel filtration chromatography was used to test whether VPS37B, TSG101, and VPS28 form higher order complexes of the size expected for human ESCRT-I. Previous studies have demonstrated that soluble human ESCRT-I complexes elute from gel filtration columns with an apparent molecular mass of ~350 kDa (22, 25). As shown in Fig. 3, exogenously expressed and tagged VPS37B also precisely eluted together with TSG101 and VPS28 in complexes of ~350 kDa. Moreover, all three proteins were present in the same complexes, as immunoprecipitation of VPS37B-FLAG from these fractions again co-immunoprecipitated TSG101 and VPS28 (data not shown). We therefore conclude that VPS37B-FLAG associates stably with the human ESCRT-I complex.

VPS37B-FLAG and endogenous TSG101 were also present in smaller complexes of overlapping but slightly different sizes (~116 and ~106 kDa, respectively). In contrast, VPS28 was found only in the ~350-kDa complex. In the absence of VPS37B overexpression, all of the endogenous TSG101 protein is normally present within the ESCRT-I complex (22), and it is therefore likely that the smaller TSG101 and VPS37B subcomplexes accumulate because VPS28 levels, although increased in the presence of elevated VPS37B, are still limiting when VPS37B is overexpressed.

VPS37B Is Trapped on Endosomal Membranes in the Absence of VPS4A ATPase Activity—Under steady-state conditions, ESCRT-I and other Class E proteins are present primarily in dispersed soluble complexes. To function in MVB cargo sorting and vesicle formation, however, these complexes must transiently associate with endosomal membranes. Following vesicle formation, these complexes then disassemble and are released from the membrane through the action of the VPS4 ATPases associated with a variety of cellular activities (25, 26, 44–50). Thus, in the absence of VPS4 ATPase activity, Class E proteins are recruited to the endosomal membrane, but become trapped on aberrant endosomal compartments (Class E compartments). This behavior can be seen for the VPS4A-GFP protein itself, where the wild-type protein was present in dis-
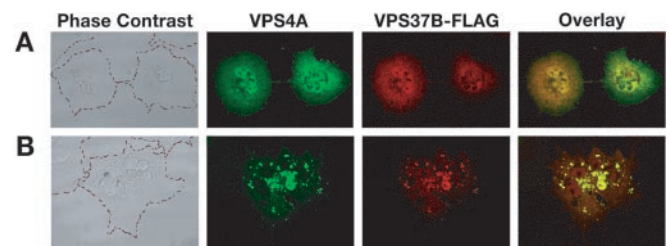
FIG. 4. **Immunofluorescent images showing the localization of VPS37B in the presence of the WT VPS4A-GFP protein (*A*) and the dominant-negative K173Q VPS4A-GFP mutant protein (*B*).** VPS4A-GFP proteins appear *green*, and VPS37B-FLAG proteins appear *red*. The *first panels* in *A* and *B* show the phase-contrast image (with cell boundaries outlined for clarity), and the *fourth panels* show an overlay of the *second* and *third panels*. Note that the dominant-negative K173Q VPS4A mutation caused both VPS4A and VPS37B-FLAG proteins to re-localize to the same sites on the Class E endosomal compartment. The "disperse" localization shown for VPS37B-FLAG in the presence of the WT VPS4A-GFP protein was seen in 90% of the images examined (47/52), whereas the "punctuate/Class E" localization shown for VPS37B-FLAG in the presence of the dominant-negative K173Q VPS4A-GFP protein was seen in 88% of the images examined (29/33).

persed soluble complexes (Fig. 4*A*, *second panel*), whereas a dominant-negative VPS4A mutant lacking the ability to bind and hydrolyze ATPase activity (K173Q) was trapped on endosomal membranes (Fig. 4*B*, *second panel*).

Analogous experiments were used to test whether VPS37B is recruited to endosomal membranes in a VPS4A-dependent fashion, as has been seen previously for the other mammalian ESCRT-I components (25). As expected, VPS37B-FLAG was distributed throughout the cell as a soluble protein when coexpressed with WT VPSP4A-GFP. This "dispersed" phenotype was observed in nearly all (90%) of the VPS4A-GFP/VPS37B-FLAG cells examined. In contrast, when VPS37B-FLAG was coexpressed together with the dominant-negative K173Q VPS4A-GFP mutant, most of the VPS37B-FLAG protein became concentrated at punctate cytoplasmic sites (88% of the cells examined). These sites corresponded to Class E compartments, as confirmed by the co-localization of VPS37B-FLAG and K173Q VPS4A-GFP (Fig. 4*B*, *fourth panel*). We therefore conclude that VPS37B behaves like the other two ESCRT-I proteins, *i.e.* the protein exists predominantly in dispersed soluble complexes under steady-state conditions, but can also associate transiently with the endosomal membrane and be released by the action of VPS4A.
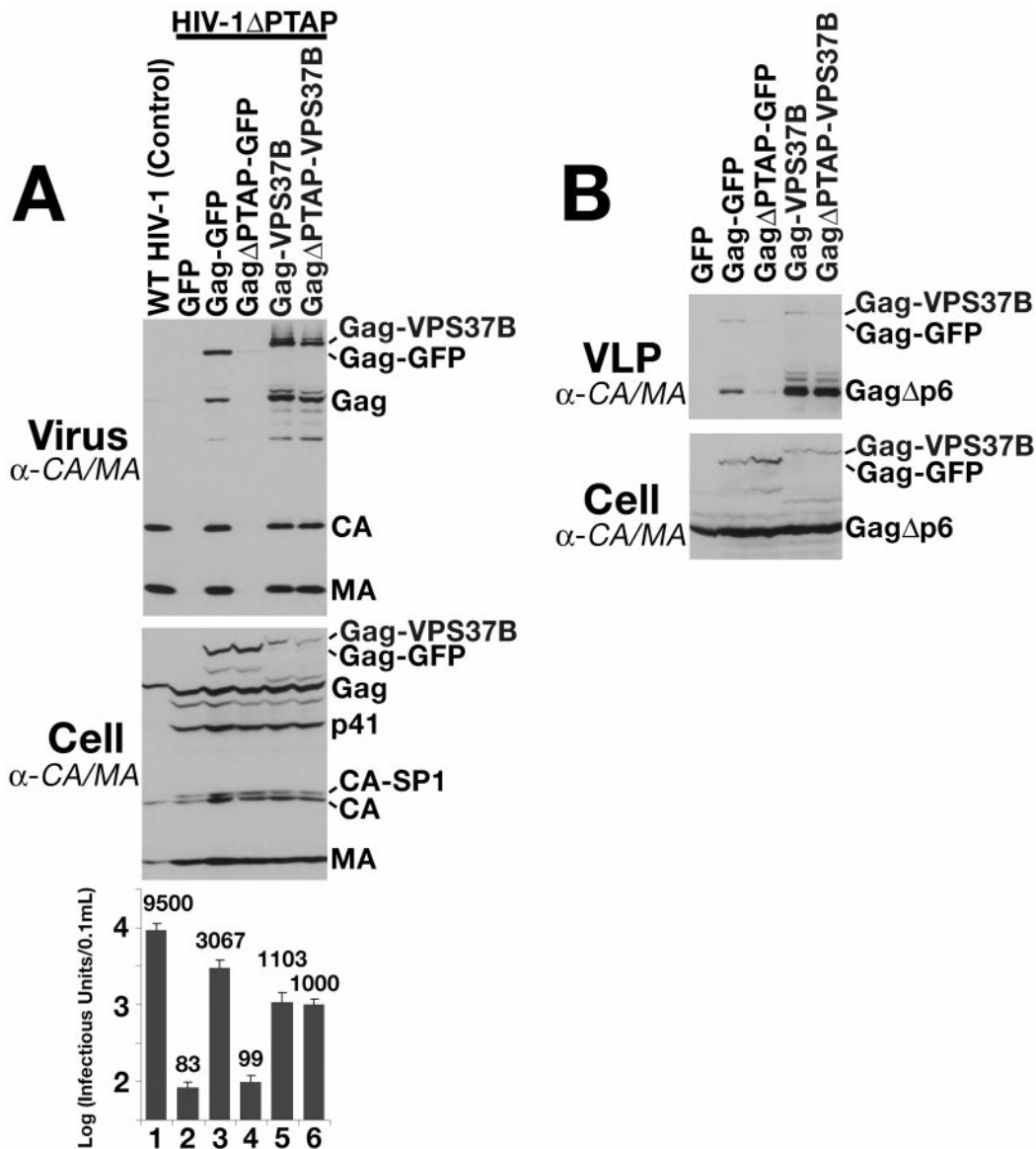
FIG. 5. **VPS37B polypeptides can rescue the release of HIV-1 constructs that lack PTAP late domains (*A*) and GagΔp6 VLPs (*B*).** *A*, VPS37B can rescue the release and infectivity of mutant HIV-1 virions that lack endogenous PTAP late domains. *Upper panel*, Western blot of HIV-1 particle-associated MA and CA proteins released from cells following transfection with 2 μg of WT HIV-1 proviral R9 plasmid (positive control; *lane 1*) or mutant proviral R9 plasmid in which the PTAP late domain of p6[Gag] was mutated to AAAA (HIV-1ΔPTAP; *lanes 2–6*). Cells were cotransfected with plasmid expressing GFP alone (negative control; *lane 2*), Gag-GFP (1 μg, positive control; *lane 3*), GagΔPTAP-GFP (1 μg, negative control; *lane 4*), Gag-VPS37B (3 μg, control for the infectivity effects of the VPS37B fusion; *lane 5*), or GagΔPTAP-VPS37B (3 μg, test for the ability of VPS37B to rescue virus budding; *lane 6*). Total vector levels were normalized to 5 μg by addition of a vector expressing GFP alone. *Middle panel*, Western blot of the same samples showing cytoplasmic Gag protein expression. Note that the bands labeled p41 and p25 are Gag processing intermediates. *Lower panel*, viral titers in the supernatants of the same samples. Titers were determined in single-cycle MAGIC assays and are plotted on a log scale. *B*, VPS37B can rescue the release of VLPs formed by Gag proteins that lack p6 (GagΔp6). *Upper panel*, Western blot of VLPs released from cells following transfection with 2 μg of plasmid expressing the HIV-1 GagΔp6 protein (*all lanes*). Cells were cotransfected with plasmid expressing GFP alone (negative control; *first lane*), Gag-GFP (positive control; *second lane*), GagΔPTAP-GFP (negative control; *third lane*), Gag-VPS37B (control for any effects of fusing VPS37B to Gag; *fourth lane*), or GagΔPTAP-VPS37B (test for the ability of VPS37B to rescue VLP release; *fifth lane*). Plasmid quantities were the same as described for *A*. *Lower panel*, Western blot of the same samples showing cytoplasmic expression of the various Gag proteins.

Human VPS37B Recruits ESCRT-I Activity—We next tested whether VPS37B can direct functional ESCRT-I activity to HIV-1 budding sites *in vivo*. Previous studies have demonstrated that ESCRT-I can support HIV-1 Gag particle release, even when recruited to sites of viral particle assembly via non-native interactions. For example, mutant retroviral Gag proteins that would otherwise be retained can bud from cells when fused directly to various proteins or domains that can recruit ESCRT-I, including ubiquitin (51), TSG101 (7), VPS28 (52), and HRS (19). In some cases, these Gag fusion proteins can even co-assemble and rescue in *trans* the release of *infec-*

tious HIV-1 virions that lack endogenous late domains, although Gag processing and virus infectivity are typically attenuated when larger proteins are fused to the C-terminal end of Gag. Thus, the heterologous rescue of virus release and infectivity provides a sensitive assay for ESCRT-I activity *in vivo*, and this approach was therefore used to test whether VPS37B can recruit functional ESCRT-I activity to sites of virus budding.

As expected, an HIV-1 construct lacking the p6[Gag] PTAP late domain (HIV-1ΔPTAP) was not released efficiently from 293T cells and was very poorly infectious (Fig. 5*A*, compare *lanes 1*

and *2*). However, HIV-1ΔPTAP release was rescued by coexpression with a wild-type Gag-GFP protein that could co-assemble with the viral Gag proteins and thereby recruit ESCRT-I activity to the sites of particle assembly (compare *lanes 2* and *3*). Virus infectivity was also substantially (although not fully) rescued by coexpression of the Gag-GFP protein (*lower panel*). The incomplete rescue of virus infectivity presumably reflected the detrimental effects of fusing GFP to the C-terminal end of the Gag protein. As expected, mutation of the PTAP late domain in the Gag-GFP construct abrogated its ability to rescue virus release and infectivity (compare *lanes 3* and *4*), confirming that rescue was late domain-dependent.

Gag-VPS37B fusion constructs could also rescue the release of HIV-1ΔPTAP, and in this case, the rescue *did not* depend upon the presence of the Gag PTAP late domain in the fusion construct (Fig. 5*A*, compare *lanes 5* and *6*). Virus infectivity was substantially rescued as well, albeit to a 3-fold lower level than in the Gag-GFP control (possibly because VPS37B (31 kDa) is even larger than GFP (27 kDa)). Gag-VPS37B fusion proteins also rescued the release of mutant Gag VLPs in *trans* (Fig. 5*B*). Indeed, GagΔPTAP-VPS37B was significantly more effective in mediating VLP release than was the Gag-GFP control construct (compare *second* and *fifth lanes*). We therefore conclude that the VPS37B polypeptide has the ability to recruit functional ESCRT-I activity *in vivo*, as assayed by its ability to rescue the release and infectivity of mutant HIV-1 and the release of mutant Gag VLPs.

Interestingly, additional higher molecular mass species were consistently seen to be associated with the different Gag species in all of the Gag-VPS37B rescue experiments. These additional species likely correspond to mono- and multiubiquitylated Gag proteins based upon the observed mobility shifts (~7 kDa larger). Thus, the VPS37B polypeptide can apparently recruit ubiquitin ligase activity (or, alternatively, reduce ubiquitin hydrolase activity (53)) to sites of virus budding.

TSG101 PTAP Binding Activity Is Essential for HIV-1 Release—We further tested whether other known TSG101/ESCRT-I interactions play important roles in HIV-1 release. To assay the functions of mutant TSG101 proteins, endogenous TSG101 was first depleted from 293T cells using siRNA. This treatment typically inhibited the release of infectious HIV-1 particles up to 50-fold. siRNA-resistant TSG101 expression constructs were then reintroduced into the cells lacking endogenous TSG101, and the ability of the ectopic TSG101 proteins (denoted TSG*) to rescue HIV-1 release and infectivity was tested. Expression of the WT TSG* protein typically rescued virus release to within 2-fold of the undepleted control. Complete rescue was not always achieved, presumably because TSG* expression levels were sometimes lower than endogenous protein levels (see "Supplemental Results" and Supplemental Fig. 1 for full details).

TSG101 depletion/rescue experiments were first used to test whether the PTAP binding activity of TSG101 is required for HIV-1 release. As noted above, the HIV-1 p6^{Gag} ^{7}PTAP^{10} motif binds in a groove in the TSG101 UEV domain, and TSG101 residues that make important contacts include Tyr^{63}, which forms one side of the Pro^{10} binding pocket, and Met^{95}, which makes extensive intermolecular hydrophobic interactions with the p6 Pro^7 and Ala^9 residues (54). Alanine substitutions of these two TSG101 residues reduce the affinity of the TSG101 UEV domain/p6 interaction partially (Y63A, 14-fold reduced) or nearly completely (M95A, 52-fold reduced) (54). These mutants were therefore used to test for a correlation between TSG101/p6 binding affinity and the ability of TSG101 to support the release of infectious HIV-1.

Infectious HIV-1 release was reduced 35-fold upon depletion
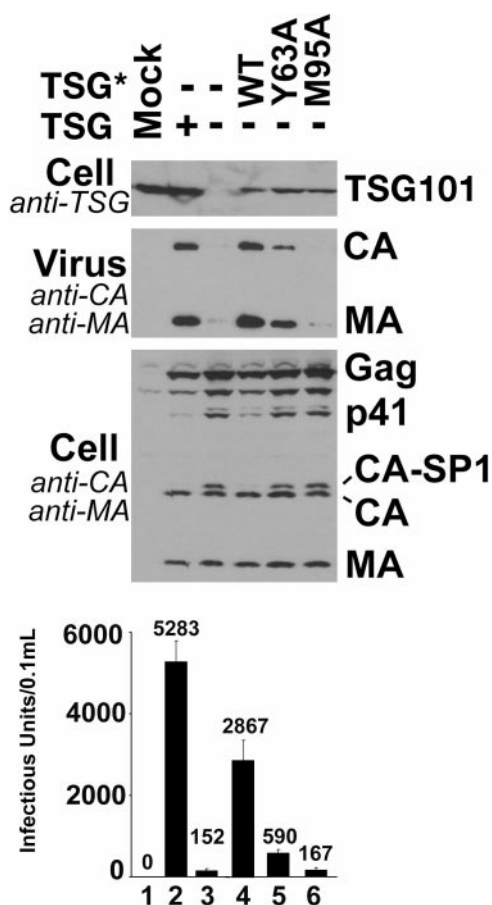
FIG. 6. **TSG101 PTAP binding is essential for HIV-1 release.** Cells were mock-transfected (*lane 1*) or transfected with the proviral R9 plasmid (*lanes 2–6*). Cells were cotransfected with siRNA duplexes to deplete TSG101 (*lanes 3–6*); with an siRNA duplex of inverted sequence (specificity control; *lane 2*); and with vectors expressing siRNA-resistant constructs (TSG*) encoding WT TSG* (*lane 4*), mutant TSG* (*lanes 5* and *6*), or no protein (control; *lane 3*). *First panel*, anti-TSG101 Western blot showing levels of endogenous TSG101 and ectopic TSG*-FLAG; *second panel*, anti-CA and anti-MA Western blot showing particle-associated CA and MA proteins released into the supernatant; *third panel*, anti-CA and anti-MA Western blot showing cytoplasmic lysates from the same samples; *fourth panel*, titers of virus released into the supernatants.

of TSG101 and rescued to within 2-fold of control levels upon reintroduction of WT TSG* (Fig. 6, compare *lanes 2–4*). In contrast, efficient HIV-1 release was not rescued by equivalent levels of either of the two TSG* PTAP motif-binding proteins, as reflected by reduced particle release, accumulation of unprocessed intracellular Gag proteins, and low infectious titers (*lanes 5* and *6*). Nevertheless, the weak binding Y63A mutant did support modest levels of virus release and infectivity (~20% of the WT TSG* control), whereas the non-binding M95A mutant failed to rescue any detectable virus release or infectivity above background levels (6). Thus, TSG101 must bind PTAP sequences with full affinity to support efficient HIV-1 release from 293T cells.

VPS28 Binds the C-terminal Domain of TSG101—Previous work in other laboratories has shown that VPS28 binds TSG101 and that the VPS28-binding site is located in the C-terminal third of TSG101 (16, 25, 29). In good agreement with these observations, VPS28 was identified 28 different times in our yeast two-hybrid library screens that employed TSG101 baits. TSG101-(317–390) was the smallest fragment that bound VPS28 in these screens, which confirmed that VPS28 binds the C-terminal region of TSG101. Similarly, pure recombinant VPS28 bound specifically to GST-TSG101-(330–
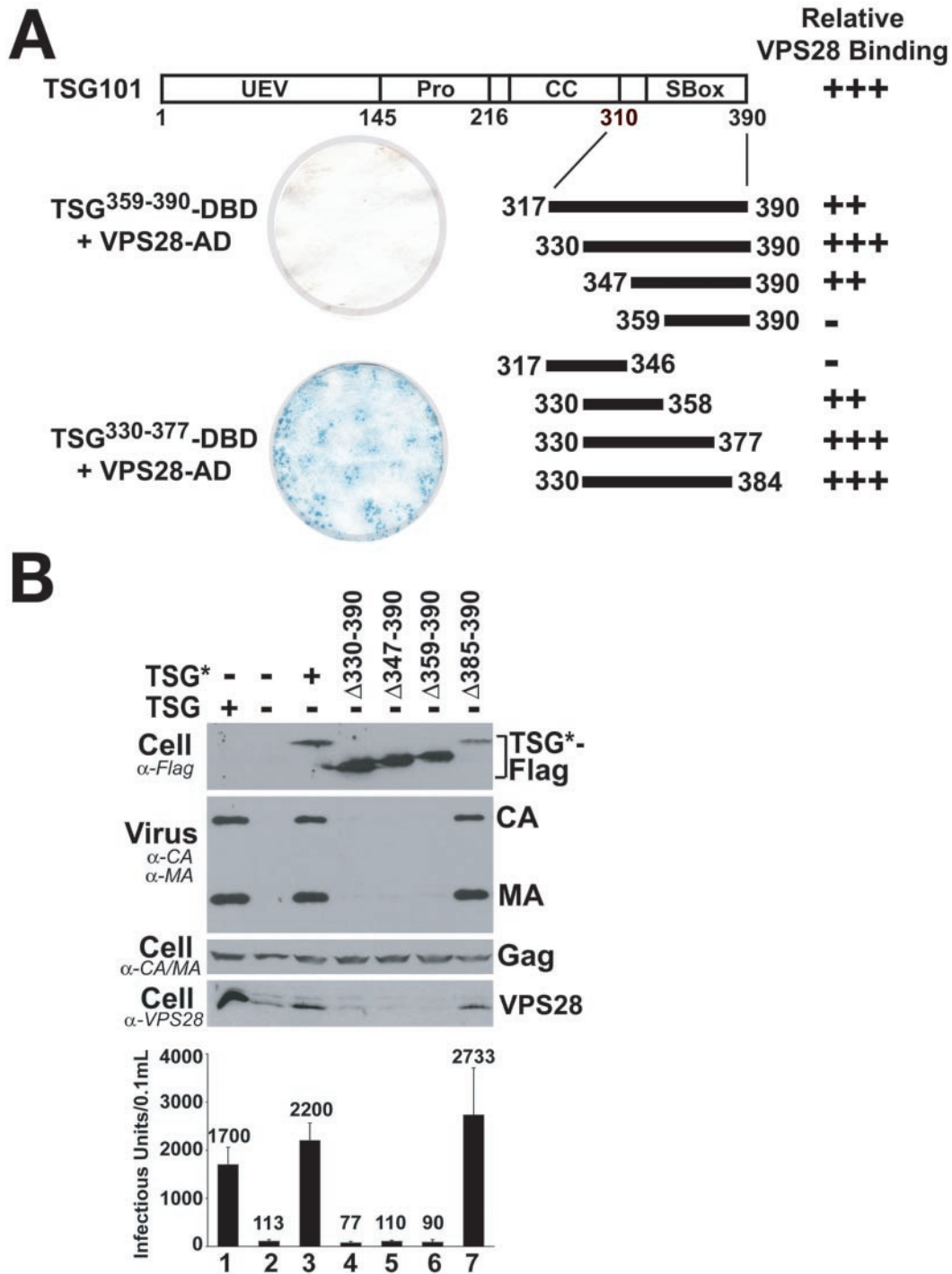
FIG. 7. **TSG101/VPS28 interactions and HIV-1 release.** *A*, the VPS28-binding site is located within the C-terminal domain of TSG101. Schematic illustrations of TSG101 and the TSG101-DBD fusion constructs are shown above. TSG101-DBD binding to VPS28-AD constructs was tested in semiquantitative liquid yeast two-hybrid assays (summarized to the right). Relative β-galactosidase activity was determined and is expressed as +++ (25–40-fold above background levels), ++ (10–25-fold above background levels), or − (undetectable binding). For reference, a filter lift assay showing the relative β-galactosidase activity resulting from the interaction between VPS28 and TSG101-(359–390) (negative) or TSG101-(330–377) (positive) is shown to the left. Note that the TSG-(330–358)-DBD fusion protein activated β-galactosidase expression in the absence of VPS28-AD. Therefore, TSG-(330–358)-AD was tested for binding to a VPS28-DBD fusion protein. *PRO*, proline-rich region; *CC*, predicted coiled-coil region; *S-Box*, steadiness box region (30). *B*, the C-terminal residues of TSG101 are essential for HIV-1 budding. Cells were transfected with the proviral R9 expression construct (*lanes 1–7*), depleted of endogenous TSG101 using a specific siRNA (denoted *TSG−*; *lanes 2–7*) or a control siRNA duplex of inverted sequence (*lane 1*), and cotransfected with plasmids expressing the indicated TSG* protein (*lanes 3–7*). *First panel*, anti-FLAG Western blot showing exogenous TSG*-FLAG levels; *second panel*, anti-CA and anti-MA Western blot of sucrose-pelleted virions showing virus release; *third panel*, anti-CA and anti-MA Western blot of cytoplasmic lysates showing Gag protein expression levels; *fourth panel*, anti-VPS28 Western blot showing endogenous VPS28 levels; *fifth panel*, viral titers in MAGIC infectivity assays of culture supernatants.

390) in "pull-down" experiments, demonstrating that the TSG101/VPS28 interaction is direct (data not shown).

Directed two-hybrid experiments and deletion analyses were used to confirm the initial binding data and to map the VPS28-

binding site more precisely. As summarized in Fig. 7, VPS28 bound well to full-length TSG101 and TSG101-(330–377). Deletion of an additional 17 residues from the N-terminal end of this construct (through residue 346) reduced VPS28 binding

significantly, and deletion of 12 more residues (through residue 359) eliminated VPS28 binding entirely. Similarly, C-terminal deletions of TSG101 through residue 359 reduced VPS28 binding, and deletions through residue 347 eliminated detectable VPS28 binding. In all cases, expression of the relevant TSG101-DBD constructs was confirmed by Western blotting with an anti-DBD antibody (data not shown). We therefore conclude that the entire VPS28-binding site spans TSG101 residues ~330–377, although ~20-residue deletions from either end of this region can be tolerated without complete loss of VPS28 binding.

TSG101 Proteins Lacking the VPS28-binding Site Fail to Rescue HIV-1 Release—We next investigated whether the TSG101/VPS28 interaction is required for HIV-1 release by testing whether a series of C-terminally truncated TSG* proteins can support virus release. As shown in Fig. 7B, the TSG*Δ330–390, TSG*Δ347–390, and TSG*Δ359–390 proteins all failed to rescue HIV-1 release and infectivity in cells depleted of endogenous TSG101 (compare *lanes 3* and *4–6*). In contrast, a TSG* protein lacking only the final six residues (TSG*Δ385–390) supported efficient HIV-1 release and infectivity (*lane 7*). The failure of the shorter TSG* proteins to rescue release was not due to their impaired expression or stability (*first panel*) or to altered HIV-1 Gag expression (*third panel*). We therefore conclude that TSG101 proteins missing all or part of the VPS28-binding site are unable to support HIV-1 release and hence that this region of TSG101 performs essential function(s) in virus budding.

Analyses of the TSG101 and VPS28 protein levels in our experiments revealed two notable trends. Endogenous VPS28 protein levels were dramatically reduced in the absence of TSG101 proteins with intact VPS28 interaction sites (Fig. 7B, *fourth panel*). This was true both in the complete absence of TSG101 (*lane 2*) and in the presence of truncated TSG* proteins lacking the entire VPS28-binding site (*lanes 4–6*). In contrast, VPS28 protein levels were higher in the presence of longer TSG* proteins that were competent to bind VPS28 (*lane 7*), and the absolute levels of VPS28 correlated well with the expression levels of the longer TSG* proteins (*lanes 3* and *7*). These observations suggest that VPS28 may be unstable when it is not bound to TSG101. The presence of cellular VPS28 also correlated well with successful HIV-1 release, as virus release was observed whenever VPS28 levels were high, but not when VPS28 levels were low.

Conversely, deletion of the VPS28-binding region *increased* the steady-state levels of exogenous TSG* (Fig. 7B, compare *lanes 3* and *7* with *lanes 4–6*). This observation is consistent with a previous report that the post-translational stability of TSG101 is negatively regulated by an element in the C-terminal region termed the "steadiness box" (residues 346–390) (30). Our data suggest the possibility that the steadiness box corresponds to the VPS28-binding site and, more generally, indicate that formation of a stable TSG101-VPS28 complex (22, 25) helps regulate the steady-state levels of both proteins.

ESCRT-I Incorporation into HIV-1 Particles—Previous studies have shown that overexpressed TSG101 can be recruited to sites of virus budding at the plasma membrane (7), that a fragment of TSG101 spanning the UEV domain can be incorporated into virus particles (8), and that EIAV Gag-VPS28 fusion proteins can recruit TSG101 into VLPs. However, these experiments did not address whether endogenous ESCRT-1 components are normally incorporated into HIV-1 particles, and this information is important for understanding precisely how the complex functions in particle release. We therefore quantitated the levels of VPS28 in highly purified HIV-1 particles.
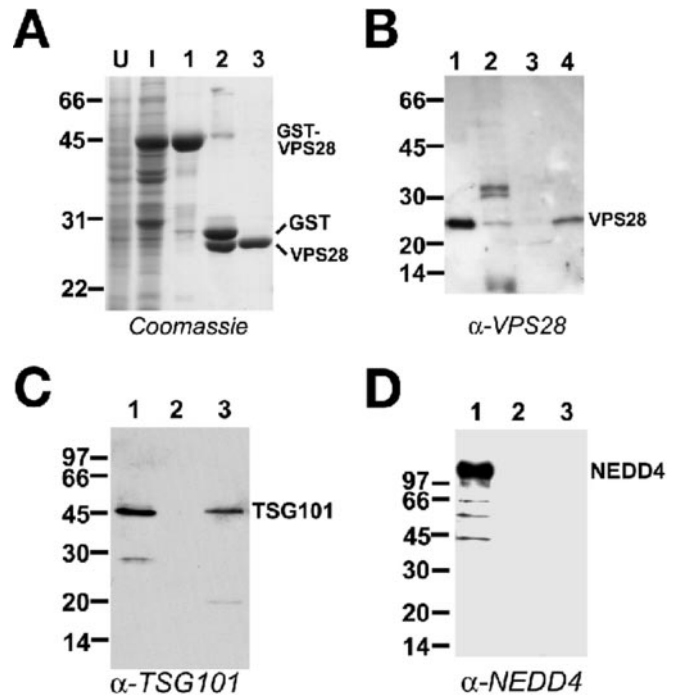
FIG. 8. **Endogenous VPS28 and TSG101 are incorporated into HIV-1 virions, whereas NEDD4 is not.** *A*, Coomassie Blue-stained gel showing the stepwise purification of recombinant VPS28 protein. *Lane U*, uninduced *E. coli* cell extract; *lane I*, induced cell extract; *lane 1*, glutathione-Sepharose-bound proteins; *lane 2*, proteins following tobacco etch virus protease cleavage; *lane 3*, purified VPS28. *B*, anti-VPS28 Western blot of 5 ng of purified recombinant VPS28 (*lane 1*), lysate from 10^5 MT-4 cells (*lane 2*), and purified HIV-1 particles corresponding to 1 μg of CA protein (*lane 4*). 1 μg of CA protein was used as a control (*lane 3*) to rule out antibody cross-reactivity in the virion preparation because VPS28 and CA have similar mobilities. Note that two cross-reacting species of higher molecular mass were observed in the crude lysate (*lane 2*). *C*, anti-TSG101 Western blot of lysate from 10^5 MT-4 cells (*lane 1*), 1 μg of recombinant CA protein (control for antibody cross-reactivity; *lane 2*), and purified HIV-1 particles corresponding to 1 μg of CA protein (*lane 3*). *D*, anti-NEDD4 Western blot of the samples described for *A*. Molecular mass markers (in kilodaltons) are shown to the left of each panel.

As anti-VPS28 antibodies were not commercially available, human VPS28 was expressed in *E. coli*, purified to homogeneity, and used to elicit anti-VPS28 antibodies in rabbits (Fig. 8A). High titer HIV-1 preparations (~10^9 infectious units/ml) containing minimal cellular contaminants were obtained from acutely infected MT-4 cells and purified by velocity gradient centrifugation (36). The purity of the sample was evaluated on silver-stained SDS gels, which showed that the Gag proteins were the main constituents and demonstrated minimal contamination with cellular proteins (data not shown) (55).

VPS28 could be detected on Western blots of pure recombinant proteins (Fig. 8B, *lane 1*), cell extracts (*lane 2*), and purified HIV-1 virions (*lane 4*). VPS28 incorporation into HIV-1 particles was confirmed in independent virus preparations and by analysis of subtilisin-treated virions, which eliminated contaminating cellular vesicles and degraded proteins on the virion exterior (56), but did not significantly reduce the amount of VPS28 in the preparations (data not shown). The absolute quantities of VPS28 present within the virion were estimated by comparing the levels of virion-associated VPS28 and CA proteins with known quantities of pure recombinant CA and VPS28 (Fig. 8A) (data not shown). In different virus preparations, between 1 and 5 ng of VPS28/μg of CA was detected, corresponding to a molar ratio of ~1:200–1000. Assuming ~5000 Gag molecules/virion (57), this implies that 5–25 molecules of VPS28 were incorporated per virion.

We also screened for HIV-1 incorporation of endogenous TSG101 and NEDD4 as well as overexpressed VPS37B-FLAG. These experiments were not quantitative, however, because we lacked the appropriate recombinant protein standards. Nevertheless, endogenous TSG101 was readily detected in both cell extracts and purified virus particles (Fig. 8*C*), indicating that the protein was specifically incorporated by HIV-1. Interestingly, both TSG101 and endogenous murine leukemia viral Gag proteins were also detected in exosomes (MVB vesicles released from cells) (58–60), which further emphasizes the similarities between viral particle release and MVB vesicle biogenesis. In contrast, NEDD4 was not detected in virions (Fig. 8*D*), despite the fact that this protein was readily detected in cell extracts and has been reported to bind TSG101 (61). Several other control proteins that partially localized to the plasma membrane were also undetectable in the purified virus preparations, confirming that these preparations were not contaminated with cellular proteins (data not shown). We therefore conclude that TSG101, like VPS28, is also specifically incorporated into HIV-1 particles, whereas NEDD4 is not. NEDD4 was detected in purified particles of Mason-Pfizer monkey virus, a virus that buds via a different late domain (PPPY) (62), reinforcing the idea that these two different late domains perform distinct functions in virus budding. Finally, we were unable to confirm that VPS37B was specifically incorporated into HIV-1 particles. Although VPS37B-FLAG was detected in purified virions, the levels appeared very low relative to the levels of overexpressed protein in the producer cells (data not shown). These observations raise the possibility that VPS37B may be lost during the process of viral assembly.

DISCUSSION

The p6^{Gag} PTAP late domain is required for efficient HIV-1 replication in most cell types, including primary cells (5). The PTAP late domain recruits TSG101, which in turn functions within a multiprotein complex (ESCRT-I) that links the virus to cellular machinery that normally functions in protein sorting and vesicle formation in the MVB (reviewed in Ref. 1). Previous studies have demonstrated that the N-terminal half of TSG101 (residues 1–249) is not required for virus budding when the C-terminal half of the protein (residues 250–390) is fused directly to the viral Gag protein (7). Thus, the N-terminal half of TSG101 appears to function primarily in recruiting the protein to the sites of virus budding, whereas the C-terminal half of the protein contains binding sites for other proteins that function in virus budding, including the other members of the ESCRT-I complex, VPS28 and VPS37. Our studies were performed with the goals of identifying human orthologs of VPS37 and testing the importance of different TSG101 interactions in HIV-1 release.

TSG101 Recruitment—TSG101 is recruited to sites of virus release, at least in part, through direct interactions with P(S/T)AP late domains found in the structural proteins from a number of different enveloped viruses, including HIV-1 (6–9). Using TSG101 depletion/replacement assays, we found an excellent correlation between the affinities of WT and mutant TSG101 proteins for the HIV-1 PTAP late domain and their ability to support HIV-1 release from 293T cells. The mutations under study presumably also affected TSG101 interactions with cellular binding partners because the TSG101 UEV domain can also bind to P(S/T)AP motifs located within a series of cellular proteins, including HRS (17–20), AIP1 (26–28), TSG101 itself (17), and VPS37B (this work). However, as TSG101 fragments lacking the UEV domain can support virus budding when fused directly to the HIV-1 Gag protein, these TSG101 UEV domain/cellular protein interactions cannot be absolutely essential for HIV budding, although they may help

to "activate" the autoinhibited full-length TSG101 protein (17–20). Hence, our experiments further confirm the functional importance of the TSG101 UEV domain/Gag P(S/T)AP interaction for HIV-1 release and indicate that any reductions in PTAP binding affinity (or levels of active TSG101 protein) can be expected to reduce viral titers. This makes the TSG101 UEV domain/PTAP motif-binding pocket an attractive target for inhibitor development.

Identification of Human VPS37 Proteins—As a necessary step in understanding how the human ESCRT-I complex functions in virus release and MVB biogenesis, we identified a series of putative human VPS37 proteins and characterized one of them, VPS37B, in detail. A number of criteria establish human VPS37B as an ortholog of yeast Vps37p, including the following. 1) The two proteins share modest but identifiable sequence similarity. 2) Both proteins bind to central/C-terminal regions of TSG101/Vps23p (16). 3) Both proteins can form stable subunits of ~350-kDa ESCRT-I complexes. 4) Both proteins become trapped on the Class E endosomal compartment in the absence of VPS4 ATPase activity. 5) VPS37B can recruit functional human ESCRT-I activity *in vivo*, as judged by its ability to support the release of infectious HIV-1 virions lacking endogenous PTAP late domains.

VPS37B belongs to a family of four human proteins that share clear sequence similarity (Fig. 1*B*), and our data demonstrate that at least one of the other proteins (VPS37A) binds TSG101. Thus, it appears that *all* of these proteins may be VPS37 orthologs. Although this idea remains to be tested experimentally, there is already precedence for this type of diversity in the mammalian MVB pathway, where other single yeast proteins appear to have diverged into multiple human proteins that perform overlapping or redundant functions (*e.g.* human VPS4A and VPS4B are both orthologs of yeast Vps4p). It will therefore be of interest to determine the relationship between the different human VPS37-like proteins, all of which appear to be widely expressed.

The sequence similarities between the different human VPS37-like proteins (and the putative *Drosophila* VPS37 protein) are strongest within an ~150-residue domain (the mod_r domain). This domain was first identified in the *Drosophila* modifier of rudimentary (Mod_r) protein, which modulates expression of the rudimentary protein (40). Although mod_r domains are found within a number of eukaryotic proteins, their functions are not well understood, and the presence of a mod_r domain therefore cannot yet be used to infer biological activity. Importantly, our mapping experiments indicate that the VPS37B mod_r domain contains the binding site for TSG101, which represents the first well characterized biochemical function for any mod_r domain.

Beyond their conserved mod_r domains, the different human VPS37-like proteins each contain unique elements that suggest interesting and unique biological functions. Specifically, VPS37B uniquely contains a PTAP motif, which is the binding site for the TSG101 UEV domain; VPS37C and VPS37D contain N- and C-terminal extensions of unknown function; and VPS37A/HCRP1 contains a putative UEV domain. This is of potential significance because other UEV domains bind ubiquitin (6, 16, 63, 64) and because ubiquitin performs a number of important roles in the MVB and HIV-1 budding pathways (1, 14, 15). However, the residues known to bind ubiquitin and PTAP sequences are not conserved in alignments of the TSG101 and VPS37A/HCRP1 UEV domains (39), and the function of this domain therefore remains to be determined experimentally.

HCRP1 was previously identified by positional cloning and shown to be down-regulated in hepatocellular carcinoma development (39). Although the protein has not been shown defini-

TSG101/ESCRT-I Functions in HIV Release

tively to act as a tumor suppressor *in vivo*, its expression is reduced in human cellular carcinomas, and reduction of VPS37A/HCRP1 levels in the human cellular carcinoma cell line BEL-7404 stimulates cell growth and enhances cell invasiveness *in vitro* (39). Similarly, reductions of TSG101 levels allowed NIH3T3 cells to grow on soft agar and to form metastatic tumors in nude mice (65), although genetic deletions of TSG101 do not induce tumor formation (66). It therefore appears that altered levels (or composition) of ESCRT-I can give rise to improperly regulated cell growth in some contexts.

Human ESCRT-I and HIV-1 Release—In addition to TSG101 and VPS37B, the human ESCRT-I complex also contains VPS28. VPS28 binds a C-terminal site on TSG101 that we have mapped to residues 330–377 and is unstable in the absence of this binding site. As in yeast, it appears that the TSG101, VPS28, and VPS37B proteins must be present in multiple copies to create a complex of ~350 kDa. Consistent with this idea, all three proteins bind to themselves in directed yeast two-hybrid experiments, suggesting that they can form homo-oligomers (Refs. 25, 26, and 29 and this work). At this stage, we cannot rule out the possibility that human ESCRT-I complexes may also contain additional proteins, and the other human VPS37 paralogs are obvious candidates.

Importantly, HIV-1 release was not supported by truncated TSG101 proteins that were missing their VPS28-binding sites (and therefore exhibited reduced VPS28 levels). This observation supports the idea that VPS28 plays a functional role in virus release and is in excellent agreement with previous work of Bieniasz and co-workers (27), who also found that VPS28 is required for HIV-1 release and identified several mutations in TSG101 residues 360–370 that inhibit both VPS28 binding and HIV-1 budding. Further evidence for a direct role for ESCRT-I in virus budding was provided by our observation that endogenous TSG101 and VPS28 were both specifically incorporated into highly purified HIV-1 particles prepared from infected T-cells, although we were unable to demonstrate specific incorporation of VPS37B. We estimate that, on average, 5–25 VPS28 molecules are incorporated into each virus particle. It is likely that virion levels of TSG101 are lower, however, as the stoichiometry of Vps28p to Vps23p in the yeast ESCRT-I complex is estimated to be ~1:6 (16).

In summary, our studies have identified human VPS37B as a new component of the human ESCRT-I complex and revealed that VPS37B can rescue the release of HIV-1 Gag proteins that lack endogenous PTAP late domains. Thus, all three known members of the human ESCRT-I complex are able to support virus release and infectivity (Refs. 7 and 52 and this work), demonstrating remarkable flexibility in the requirements for viral entry into the MVB biogenesis pathway.

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