TSG101/Mammalian VPS23 and Mammalian VPS28 Interact Directly and Are Recruited to VPS4-induced Endosomes*

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Class E vacuolar protein sorting (vps) proteins are required for appropriate sorting of receptors within the yeast endocytic pathway, and most probably function in the biogenesis of multivesicular bodies. We have identified the mammalian orthologue of Vps28p as a 221-amino acid cytosolic protein that interacts with TSG101/mammalian VPS23 to form part of a multicomponent complex. Co-immunoprecipitation and cross-linking experiments demonstrated that hVPS28 and TSG101 interact directly and that binding requires structural information within the conserved C-terminal portion of TSG101. TSG101 and hVPS28 are predominantly cytosolic. However, when endosomal vacuolization was induced by the expression of a dominant-negative mutant of another class E vps protein, human VPS4, a portion of both TSG101 and hVPS28 translocated to the surface of these vacuoles. We conclude that TSG101 and its interacting components are directly involved in endosomal sorting.

Cell surface receptors are internalized in endocytic vesicles and enter the early endosome, where they are sorted. Many receptors return to the cell surface to engage in further rounds of ligand uptake, whereas other receptors are targeted via the multivesicular body (MVB)/late endosome for degradation in the lysosome (1). The molecular basis for selection of these receptors and their transfer to the MVB remains poorly characterized. Studies in yeast have, however, identified a number of gene products that are required for post-endosomal sorting. vps (vacuolar protein sorting) mutants are defective in delivering newly synthesized hydrolases to the vacuole (the yeast lysosome) (2). Among these, class E vps mutants are characterized by the formation of an exaggerated endocytic compartment (3). At least 15 class E vps mutants have been identified in yeast. It has been proposed that they act at a common step in endosomal sorting, and this has been borne out by several studies demonstrating physical and/or genetic interactions between them.

The class E compartment contains endocytosed markers, as well as precursors of vacuolar hydrolases and markers normally associated with the trans Golgi (4–8). Because proteins are retained in the yeast trans Golgi by a mechanism that involves their transport to and retrieval from the endocytic pathway (8), class E proteins appear to be important for sorting material bound for the vacuole away from proteins that cycle through the endocytic system. Indeed, recent data are consistent with class E proteins participating in the formation of MVB (9).

To further our understanding of the function of class E proteins on the mammalian endocytic pathway, we have generated specific reagents for a number of close structural mammalian homologues of yeast class E vps proteins. We (10), and others (11), have shown that mammalian orthologues of the AAA (ATPases Associated with cellular Activities)-type ATPase, Vps4p, are required to maintain the morphological and functional organization of the endocytic pathway. Like yeast Vps4p (12), mammalian (m)VPS4 (and the closely related SKD1 (13)) couples its ATPase activity to a cycle of binding to and release from endosomal membranes (10, 11). Although the immediate substrates for mVPS4 have yet to be identified, our data are consistent with mVPS4 acting to release other soluble class E components from endosome-associated multicomponent complexes and thus recycle them for further rounds of sorting.

In this study we have examined the interactions of TSG101, previously identified as the mammalian orthologue of Vps23p/Stp22p (14, 15), another class E vps protein. TSG101, like Vps23p/Stp22p, positively regulates the lysosomal degradation of cell surface proteins, including mitogenic receptors (14, 15). Tsg101 was identified originally as a tumor susceptibility gene by an assay for cellular transformation upon random gene disruption (16), although its importance as a tumor suppressor gene is still debated. A number of studies have reported the presence of aberrant splice variants of TSG101 in specific human tumors (16, 17), although the significance of these findings has been disputed (18, 19).

Although the sorting defect associated with TSG101-deficient cells could underlie the susceptibility of these cells to transformation, limited evidence is available to explain how TSG101 acts to regulate mitogenic receptor down-regulation. The TSG101 peptide sequence was first interpreted to contain two DNA-binding motifs, perhaps indicative of a transcription factor (16). However, the existence of the DNA-binding motifs has not been substantiated in subsequent analyses (20, 21). Notwithstanding this, recent reports have linked TSG101 to transcriptional regulation and have provided evidence that TSG101 can act in vitro as a transcriptional suppressor (22, 23). Therefore, TSG101 could act indirectly to aid receptor degradation by modulating the expression of specific protein(s),
themselves involved in endosomal trafficking. Alternatively, TSG101 might interact with a number of other class E vps proteins to form a sorting complex on the surface of the sorting endosome. Here, it might function to recognize cargo destined for inclusion in the multivesicular body, or provide a structural component for the sorting event.

As part of our studies we have identified a mammalian orthologue of a further class E vps protein, Vps28p. We now demonstrate that human VPS28 (hVPS28) interacts directly with TSG101. Moreover, both TSG101 and hVPS28 can be recruited to early endosome membranes by co-expression of a dominant-negative mutant of VPS4, consistent with a direct role for the TSG101 complex in receptor sorting.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Media—**Escherichia coli strain XL1Blue (Stratagene) was used for maintenance, sequencing, and mutation of plasmids. Luria-Bertani medium was used for growth of E. coli cells. For selection of plasmids, 100 µg/ml ampicillin or 25 µg/ml kanamycin was added to the media.

**Antibodies—**Proteins tagged with myc/His6 were detected using mouse monoclonal antibody 9E10 (Sigma) or anti-polyhistidine (Sigma). Anti-TSG101 was from AbCam, Cambridge, UK. An anti-hVPS28 antibody was prepared in rabbit, with His6 recombinant hVPS28 used as antigen. Immunizations were performed by Diagnostics Scotland (Carluke, Lanarkshire). Antibody was affinity-purified using glutathione-S-transferase-tagged hVPS28.

**DNA Manipulations—**Standard protocols for recombinant DNA manipulation were used (24). The ABI PRISM Big dye terminator system was used for DNA sequencing, and mutagenesis was performed using the QuikChange (Stratagene) method according to the manufacturer’s instructions, or by cassette mutagenesis (see below). Mutagenesis and subcloning constructs were confirmed by DNA sequencing.

A human cDNA EST clone from a Soares sesenecis fibrolast (NhHSF) library (Clone ID 1331807) was obtained from the IMAGE consortium (25) in vector pT7T3D, with a modified polylinker. The EST sequence had amino acid similarity to the C-terminal region of yeast VPS28, and was designated hVPS28 (human VPS28; GenBank® accession number AF316887). Multiple sequence alignments were performed using the BCM search launcher and the ClustalW 1.8 program (26, 27), and graphs were performed using the BOXSHADE server.

The cDNA insert for hVPS28 was subcloned into two mammalian expression vectors pEGFP (Clontech) and pcDNA3.1myc/His (Invitrogen). These vectors were designed to express hVPS28 with an enhanced green fluorescent protein (eGFP) tag at the N terminus of the protein (hVPS28-eGFP) or a myc/His6 tag at the C terminus (hVPS28-pcDNA3.1-myc/His). Subsequently, the myc or His6 epitopes were deleted from hVPS28-pcDNA3.1-myc/His, and therefore, these vectors were expressed hVPS28 with a His6 or myc tag alone (hVPS28-pcDNA3.1-His and hVPS28-pcDNA3.1-myc). For prokaryotic expression, hVPS28 was subcloned into pGEX (Amersham Pharmacia Biotech) and pTrcHis (Invitrogen) vectors by standard methods.

Plasmid pBluescript SKII minus (pBS, Stratagene) was used as a cloning vehicle. The mTSG101 gene was subsequently subcloned into pcDNA3.1myc/His, with the stop codon still present before the myc/His6 tag, and was named mTSG101-pcDNA3.1. The stop codon was then deleted to allow expression of mTSG101 with an in-frame myc/His6 tag at the C-terminal end. An HA epitope tag was introduced at the N terminus of mTSG101-pcDNA3.1, creating vector HA-mTSG101-pcDNA3.1, designed to express mTSG101 with an N-terminal HA epitope tag. TSG101 domain deletions were performed by introducing restriction enzyme sites as indicated in Fig. 4A. The DNA was cut using pair-wise combinations of restriction enzymes, and then religated.

**Secondary Structure Prediction—**Secondary structure prediction was determined using a variety of secondary structure prediction methods via the PredictProtein Sequence Analysis server. These included DPM (28), DSC (29), GOR (30–33), MLRC (34), PHD (35), Predator (36), SIMPA96 (37), and SOPM (38, 39).

**Multiple Tissue cDNA Analysis—**A human multiple tissue cDNA panel (CLONTECH) was probed using hVPS28-specific primers according to the manufacturer’s instructions, using good PCR practices, and utilizing touchdown PCR to increase the specificity of the reaction. The levels of PCR products were also analyzed before the reactions reached their plateau (i.e. before saturation), as determined by taking samples of each PCR product after varying cycle times. This ensured that the relative abundance of target in each tissue could be directly compared. The primers used to amplify the hVPS28 gene were located: (i) just after the initiation codon, and (ii) in the 3’-untranslated region. A primer in the 5’-untranslated region was used to define the length of the cDNA and to amplify a related member of the same gene family. The sequences of the primers were (i) cat ggg atc cca gcc agc ceg cgc ata gg and (ii) gtg gat gac cac ggc ctg tgt ggc gg. The multiple tissue panel used had been normalized using the mRNA expression levels of several housekeeping genes, allowing use of the panel to estimate the relative abundance of hVPS28 expression. The PCR products were analyzed after the reactions reached its plateau, by monitoring PCR products at five time points during thermal cycling. PCR products were run in parallel with DNA size markers (MBI Fermentas).

**Cell Culture—**All cells were grown in Dulbecco’s modified essential medium (Dulbecco’s modified Eagle’s medium; Life Technologies, Inc.) containing 10% fetal calf serum. For fluorescence studies, cells were transfected with 30% confluent using the lipid-based FuGene method (Roche Molecular Biochemicals). Controls of expression vectors without insert and FuGene alone were used. Cells grown on glass coverslips were fixed 18–24 h post-transfection using methanol at −20 °C and mounted using ProLong mount (Molecular Probes). Cells were examined using a Leica confocal microscope. For large scale transfections, COS cells were passaged in medium containing HyClone fetal calf serum and transfected using the Profection CaPO4-based reagent (Promega).

**Cell Extracts—**Cells were washed in homogenization buffer (10 mM triethanolamine, pH 7.4, 1 mM MgCl2, 250 mM sucrose) and harvested by scraping. The suspended cells were lysed by passing 20 times through a fine microipette tip, and postnuclear supernatants were prepared by centrifugation for 5 min at 1000 g. Where appropriate, a protein concentration of each sample was determined by the Bradford assay. For gel filtration experiments, cytosols were prepared by centri fuging cell extracts at 400,000 × g for 15 min in a TL100 bench-top ultracentrifuge (Beckman). Samples were applied to a 2.4-ml Superose 12 column equilibrated in 20 mM Hepes, pH 7.4, 10 mM KOAc, 1 mM MgCl2, and fractionated on an Amersham Pharmacia Biotech Smart System. Proteins were precipitated using chloroform/methanol (40) and processed for SDS-PAGE and immunoblotting.

**Immunoprecipitation and Cross-Linking—**In vitro translations were performed using standard procedures and reticulocyte lysates from Promega. Each translation typically contained 1 µg of DNA and 60 µCi of [35S]methionine. Translations were terminated by addition of 1 mM cycloheximide. The specificity of translation was confirmed by SDS-PAGE and phosphorimaging. For generation of hTDBA-lysyl [35S]protein, TDBA-modified lysyl tRNA was prepared as described previously (41) and used to supplement in vitro translation reactions at the level of 40 pmol per 25 µl of translation mix (42). For native immunoprecipitations, 5–20 µl translation mix was diluted to 200 µl in immunoprecipitation buffer (20 mM Hepes-NaOH, pH 7.4, 100 mM NaCl, 1% (v/v) Triton X-100) containing 1 µl of ascites and incubated on ice for 3–4 h. 15 µl of protein G-agarose beads (Zymed Laboratories Inc.) was added, and the incubation continued overnight with constant mixing, prior to washing.

For cross-linking experiments, samples containing TDBA-lysyl translation products were diluted at least 5-fold and irradiated for 5 s using a UV lamp (SpotCure, Ultra Violet Products Ltd., Cambridge, UK). Native immunoprecipitations were performed as normal. For secondary structure predicting immunoprecipitations, protein G-agarose beads containing bound antigen were incubated with 30 µl of denaturing buffer (100 mM Tris-HCl, pH 7.6, 140 mM NaCl, 1% (w/v) SDS) for 5 min at 95 °C. After addition of 170 µl of immunoprecipitation buffer, samples were mixed on ice and centrifuged. The resulting supernatants were incubated with a second antibody for 4 h at 4 °C. Protein G-agarose beads were added, and samples were treated as for native immunoprecipitations.

**RESULTS AND DISCUSSION**

**Human VPS28—**The nucleotide sequence of the EST clone with EMBL accession number W47604 was determined. Sequence comparisons at the amino acid level with those on the data bases indicated that the cDNA encoded a human homologue of yeast Vps28p (GenBank® accession number U50630), and was designated hVPS28 (Fig. 1A). Comparison with other proteins on the data base indicated that homologues of Vps28p...
Mammalian Class E vps Proteins

FIG. 1. Characterization of human VPS28. A, nucleotide and deduced amino acid sequence of the coding region of hVPS28 cDNA. An in-frame stop codon is underlined, and the ATG conforms to a Kozak consensus. B, sequence comparison between VPS28 amino acid sequence from various species. H. sapiens (Hs), D. melanogaster (Dm), C. elegans (Ce), A. thaliana (At), S. cerevisiae (Sc), and S. pombe (Sp). Identical residues are highlighted in black; similar residues are gray. C, tissue distribution of hVPS28 mRNA. Comparison of hVPS28 mRNA expression in various tissues using a multiple tissue cDNA panel. A PCR product of ~750 bp was detected in all human tissues examined. D, localization of hVPS28. hVPS28-myc/His6 was transfected into NRK cells and visualized by staining with anti-myc antibody. E, same as D, but stained with affinity-purified anti-hVPS28. F, the affinity-purified polyclonal antibody against recombinant hVPS28 was used to probe extracts from COS cells using a final antibody concentration of 2 μg/ml. G, affinity-purified anti-VPS28 was used to detect VPS28 in untreated NRK cells.
are also found in Drosophila melanogaster (SPTREMBL: Q97359), Caenorhabditis elegans (SPTREMBL: Q9NA26), Arabidopsis thaliana (SPTREMBL: Q9S977), and Schizosaccharomyces pombe (SPTREMBL: Q10872) (Fig. 1B). Overall comparison of the VPS28 protein of the six species revealed an overall 34% sequence similarity. Saccharomyces cerevisiae Vps28p (6) shows 28% amino acid sequence identity with hVPS28 with a further 38% similarity, giving an overall sequence similarity of 66%. Human VPS28 is most closely related to that of D. melanogaster. The major difference in protein sequence between S. cerevisiae VPS28 protein and that of other species is an insertion of around 20–35 amino acids in the middle of the protein. These proteins are hydrophilic with no hydrophobic regions predicted to span a lipid bilayer. No significant similarities to known protein sequence motifs were revealed by searching the Prosite pattern, Blocks, ProDom, or Prints data bases.

A multiple tissue cDNA panel was screened using PCR primers to the hVPS28 5′ coding region and 3′-untranslated region (Fig. 1C). The hVPS28 mRNA is ubiquitous, because a PCR product of around 750 bp was detected in all human tissues examined (brain, heart, kidney, liver, lung, pancreas, placenta, and skeletal muscle). No size variants were detected even when saturation of the PCR reactions occurred (data not shown), indicating splice variants of hVPS28 are not expressed.

To determine the cellular localization of mammalian VPS28, a mammalian expression vector containing hVPS28 fused to a myc/His<sub>6</sub> tag at the C terminus was used. As shown in Fig. 1D, hVPS28 expressed in NRK cells was exclusively cytosolic. An affinity-purified antibody that specifically recognized hVPS28 by Western blot (Fig. 1F) also recognized the expressed hVPS28 (Fig. 1E). Staining untransfected cells with anti-VPS28 antibody revealed that endogenous VPS28 was likewise distributed throughout the cytoplasm, though the staining was typically more granular than for expressed hVPS28 (Fig. 1G). At present, we do not know why the pattern of endogenous VPS28 staining differs from that of expressed VPS28. Fractionation experiments (data not shown) indicate that endogenous VPS28 is largely soluble. However, it is possible that much of this pool is lost during methanol fixation for immunofluorescence staining. The particulate VPS28 staining did not co-localize with markers for early endosomes, late endosomes, or lysosomes (data not shown).

hVPS28 Interacts Directly with TSG101/mVPS28—Evidence from yeast suggests that many class E vps proteins interact with each other to form multiprotein complexes. To examine whether hVPS28 binds to other mammalian class E vps proteins, <sup>35</sup>S-labeled hVPS28 was translated in vitro. Translations were terminated by addition of cycloheximide. Samples of TSG101-myc/His<sub>6</sub> (lane 4) or hVPS28-myc/His<sub>6</sub> (lane 5) were immunoprecipitated with anti-His<sub>6</sub> antibody and analyzed by SDS-PAGE and phosphorimaging. B, <sup>35</sup>S-labeled TSG101-His<sub>6</sub> (top panel) was incubated alone (lanes 1 and 2, 7, and 8), with unlabeled TDBA-TSG101-myc (lanes 3 and 4, 9, and 10), or with unlabeled TDBA-hVPS28-myc (lanes 5 and 6, 11, and 12). Samples were not treated (odd lanes) or UV irradiated (even lanes) and immunoprecipitated with anti-His under native conditions (lanes 1–6) and subsequently by anti-myc under denaturing conditions (lanes 7–12). <sup>35</sup>S-Labeled mVPS28-His<sub>6</sub> (bottom panel) was incubated alone (lanes 1 and 2), with unlabeled TDBA-hVPS28-myc (lanes 3 and 4), or with TDBA-TSG101-myc (lanes 5 and 6). Samples were not treated (odd lanes) or UV irradiated (even lanes) and immunoprecipitated with anti-myc under denaturing conditions.

FIG. 2. hVPS28 interacts directly with TSG101. A, TSG101-myc/His<sub>6</sub> (lane 1) or hVPS28-myc/His<sub>6</sub> (lane 2), and hVPS28-His<sub>6</sub> (lane 3) were translated in vitro. Translations were terminated by addition of cycloheximide. Samples of TSG101-myc/His<sub>6</sub> (lane 4) or hVPS28-myc/His<sub>6</sub> (lane 5), TSG101-His<sub>6</sub> (lane 6), and hVPS28-His<sub>6</sub> combined with TSG101-myc/His<sub>6</sub> (lane 7) were incubated for a further 1 h at 30 °C. Samples were immunoprecipitated with anti-myc antibody and analyzed by SDS-PAGE and phosphorimaging. B, <sup>35</sup>S-labeled TSG101-His<sub>6</sub> (top panel) was incubated alone (lanes 1 and 2, 7, and 8), with unlabeled TDBA-TSG101-myc (lanes 3 and 4, 9, and 10), or with unlabeled TDBA-hVPS28-myc (lanes 5 and 6, 11, and 12). Samples were not treated (odd lanes) or UV irradiated (even lanes) and immunoprecipitated with anti-His under native conditions (lanes 1–6) and subsequently by anti-myc under denaturing conditions (lanes 7–12). <sup>35</sup>S-Labeled mVPS28-His<sub>6</sub> (bottom panel) was incubated alone (lanes 1 and 2), with unlabeled TDBA-hVPS28-myc (lanes 3 and 4), or with TDBA-TSG101-myc (lanes 5 and 6). Samples were not treated (odd lanes) or UV irradiated (even lanes) and immunoprecipitated with anti-myc under denaturing conditions.

hVPS28 contains a monovalent UV-activated cross-linking moiety (41, 43, 44). Samples were treated with or without UV irradiation and immunoprecipitated under native conditions with anti-His antibody. No high molecular weight cross-linking adducts were observed upon UV irradiating <sup>35</sup>S-labeled TSG101-His<sub>6</sub> alone or <sup>35</sup>S-labeled TSG101-His<sub>6</sub> combined with TDBA-TSG101-myc (Fig. 2B, top panel, lanes 2 and 4). However, when <sup>35</sup>S-labeled TSG101-His<sub>6</sub> was combined with TDBA-hVPS28-myc, two high molecular weight radiolabeled adducts were detected upon UV irradiation (Fig. 2B, top panel, lane 6, asterisks). No adducts were obtained without UV irradiation (Fig. 2B, top panel, lane 5). Furthermore, the adducts were confirmed to contain hVPS28-myc by immunoprecipitation with anti-myc antibody under denaturing conditions (Fig. 2B, top panel, lane 12, asterisks).

The same high molecular weight adducts were observed when <sup>35</sup>S-labeled hVPS28-His<sub>6</sub> was combined with unlabeled TDBA-TSG101-myc (Fig. 2B, bottom panel, lane 6, asterisks) but not when <sup>35</sup>S-labeled hVPS28-His<sub>6</sub> was incubated alone or with TDBA-myc-hVPS28 (Fig. 2B, bottom panel, lanes 2 and 4). Together, these results confirm that TSG101 and hVPS28 bind directly to each other but suggest that TSG101 and hVPS28 homodimers are not formed. Likewise, no evidence for homodimerization of TSG101 or hVPS28 was obtained when <sup>35</sup>S-labeled His<sub>6</sub>- and myc-tagged proteins were co-translated and immunoprecipitated with anti-myc antibody (data not shown). The apparent molecular masses of ~80 and 110 kDa for the
of hVPS28-migrated to a position consistent with a monomer, the presence of the complex (Fig. 3), incubated with immobilized anti-hVPS28 antibody (Fig. 3); although the start codon of the S. pombe gene has not been conclusively identified. The similarities between each of these proteins was calculated using the ClustalW program (26), and phylogenetic analysis was performed to create an unrooted tree (Fig. 4B). The tree indicates that human and mouse TSG101 are most closely related to each other (95% identical; 99% similar) and then are most similar to C. mydas caranigra TSG101 (89% identical; 97% similar). A protein closely related to TSG101 is also found in D. melanogaster (Accession number Q9VVA7). This protein lacks the first part of the ubiquitin-conjugating (UbC) domain (see Fig. 4C). The portion of the UbC domain missing is predicted to delete the motif conserved in inactive E2 UbC proteins, and the first N-terminal loop of the active UbC region, a leucine zipper, and a C-terminal helical domain. The functional relationship of this protein to TSG101 is unknown.

The C-terminal portion of TSG101 has the potential to form an amphipathic alpha helical domain (see Fig. 4D), as determined using a variety of secondary structure prediction methods via the Network Protein Sequence Analysis server. The C-terminal alpha helical domain is the most highly conserved domain within TSG101 (15). We have also found that the coiled coil-forming region of mammalian TSG101 has the potential to form a more extensive leucine zipper than first described, extending for seven helical turns (Fig. 4D). Leucine zippers share a characteristic seven-amino acid (heptad) repeat (abcedeg)n with hydrophobic residues at the first (a) and fourth (d) positions. Unlike traditional leucine zippers, TSG101 has a preponderance of hydrophobic residues at position e as well as position d. Other nontraditional leucine zippers have been identified, including one within the HIV-1 gp41 protein that has hydrophobic residues at positions e and g (45). The repeated leucines in position a are substituted with isoleucine and valine in some TSG101 orthologues, as found in other coiled coil structures (46, 47). The coiled coil-forming potential of this region was indicated by use of the COILS computer program (48). As well as in mammalian TSG101, the putative seven turn zipper is conserved in C. mydas caranigra, S. cerevisiae, S. caribergensis, and A. thaliana, whereas it is somewhat shorter in S. pombe and C. elegans.

hVPS28 Binds to the Conserved C Terminus of TSG101—To investigate which region(s) of TSG101 is/are required for its interaction with hVPS28, domain-deletion mutants of TSG101 were generated by engineering restriction sites into its coding sequence. Approximately equal quantities of unlabeled, in vitro translated myc-tagged TSG101 were incubated with 35S-labeled hVPS28-His6 and immunoprecipitated with anti-myc. As shown in Fig. 5A, TSG101 lacking the UbC domain or proline-rich region interacted with hVPS28 as well as did full-length TSG101. In contrast, TSG101 lacking the C-terminal helical domain was completely unable to bind hVPS28, whereas TSG101 lacking the leucine zipper domain bound to hVPS28 but with reduced ability. The C-terminal portions of TSG101 were sufficient to account for its binding to hVPS28, because a construct containing the leucine zipper and C-terminal helical domains alone bound hVPS28 to the same extent as did full-length TSG101 (Fig. 5B). Indeed, the C-terminal helical domain of TSG101 alone bound to hVPS28 about 80% as efficiently as full-length TSG101. Hence, the most conserved region within TSG101 is responsible for its interaction with

![Fig. 3. Cytosolic TSG101 binds to hVPS28.](image)

A, cytosol from COS cells was prepared as described under “Experimental Procedures” and was fractionated by size exclusion chromatography on a 2.4-ml Superose 12 column. 50-μl fractions were analyzed for TSG101 and hVPS28 content. Migration of molecular mass markers is indicated: 670 kDa, thyroglobulin; 250 kDa, catalase; 80 kDa, transferrin; 65 kDa, bovine serum albumin. B, cytosol from COS cells expressing hVPS28-myc was fractionated and analyzed with anti-TSG101 and anti-myc antibodies. C, cytosol from COS cells was incubated with protein G-agarose beads containing preabsorbed anti-VPS28. Control beads were incubated with unrelated sheep IgG. Samples were cleared by centrifugation and supernatants analyzed for TSG101 content.

TSG101-hVPS28 adducts are consistent with formation of both TSG1011-hVPS281 and TSG1011-hVPS282 species. However, cross-linking products may run aberrantly on SDS-PAGE, so that other interpretations of these data are possible.

To provide further evidence that cytosolic TSG101/mVPS23 and hVPS28 interact, a high speed supernatant from COS cells was fractionated by size exclusion chromatography and analyzed by Western blotting. TSG101 migrated as a single species with an apparent molecular mass of 350–400 kDa, consistent with previous findings (15), and the migration of hVPS28 was precisely coincident with TSG101 (Fig. 3A). Moreover, overexpression of hVPS28-myc affected the stability of the TSG101 complex (Fig. 3B). Although all of the detectable hVPS28-myc migrated to a position consistent with a monomer, the presence of hVPS28-myc resulted in a spread of TSG101 molecular mass between −100 and 400 kDa (Fig. 3B). It remains unclear whether TSG101 complexes are destabilized within the cell by excess hVPS28-myc, or whether the reduced stability is only apparent after dilution of the complex during chromatography. Finally, TSG101 was substantially depleted from cell extracts incubated with immobilized anti-hVPS28 antibody (Fig. 3C), confirming that most, if not all, cellular TSG101 is complexed to hVPS28. It is likely that the 350- to 400-kDa complex contains additional components involved in endosomal sorting, because we have found no evidence that either TSG101 or hVPS28 can homodimerize.

Analysis of the tsg101 Gene Product—The mammalian tsg101 gene product was originally described as having a coiled coil domain encompassing a leucine zipper of four helical turns and a proline-rich region (16). Subsequent analysis noted the presence of an N-terminal region with significant homology to ubiquitin-conjugating enzymes (20, 21). The coiled coil domain interacts with nuclear proteins (22, 23) and is predicted to interact with the cytosolic protein stathmin (58, 59). Recently, the yeast gene STP22 was found to be orthologous to human and mouse tsg101 (15), demonstrating that tsg101 is highly conserved.

Our analyses of the protein sequence of mammalian TSG101 have identified further features of this protein. In addition to orthologues in Homo sapiens, Mus musculus, and S. cerevisiae, we have found potential orthologues in C. elegans, Saccharomyces caribergensis pastorianus, S. pombe, Chelonia mydas caranigra (Green sea turtle) and A. thaliana (Mouse-ear cress) (Fig. 4A), although the start codon of the S. pombe gene has not been conclusively identified. The similarities between each of these proteins was calculated using the ClustalW program (26), and phylogenetic analysis was performed to create an unrooted tree (Fig. 4B). The tree indicates that human and mouse TSG101 are most closely related to each other (95% identical; 99% similar) and then are most similar to C. mydas caranigra TSG101 (89% identical; 97% similar). A protein closely related to TSG101 is also found in D. melanogaster (Accession number Q9VVA7). This protein lacks the first part of the ubiquitin-conjugating (UbC) domain (see Fig. 4C). The portion of the UbC domain missing is predicted to delete a motif conserved in inactive E2 UbC proteins, and the first N-terminal loop of the active UbC (21). After the truncated UbC region, this protein retains the same overall domain structure to TSG101 with a proline-rich region, a leucine zipper, and a C-terminal helical domain. The functional relationship of this protein to TSG101 is unknown.

The C-terminal portion of TSG101 has the potential to form an amphipathic alpha helical domain (see Fig. 4D), as determined using a variety of secondary structure prediction methods via the Network Protein Sequence Analysis server. The C-terminal alpha helical domain is the most highly conserved domain within TSG101 (15). We have also found that the coiled coil-forming region of mammalian TSG101 has the potential to form a more extensive leucine zipper than first described, extending for seven helical turns (Fig. 4D). Leucine zippers share a characteristic seven-amino acid (heptad) repeat (abcedeg)n with hydrophobic residues at the first (a) and fourth (d) positions. Unlike traditional leucine zippers, TSG101 has a preponderance of hydrophobic residues at position e as well as position d. Other nontraditional leucine zippers have been identified, including one within the HIV-1 gp41 protein that has hydrophobic residues at positions e and g (45). The repeated leucines in position a are substituted with isoleucine and valine in some TSG101 orthologues, as found in other coiled coil structures (46, 47). The coiled coil-forming potential of this region was indicated by use of the COILS computer program (48). As well as in mammalian TSG101, the putative seven turn zipper is conserved in C. mydas caranigra, S. cerevisiae, S. caribergensis, and A. thaliana, whereas it is somewhat shorter in S. pombe and C. elegans.
**Fig. 4. Analysis of the tsg101 gene product.**

**A,** sequence comparison between TSG101 amino acid sequence from various species. A. thaliana (At; BAB03147); C. mydas caranigra (Cc; AAF87776); S. pombe (Sp; Q9UTP6); M. musculus (Mm; U52945); H. sapiens (Hs; U82130); S. cerevisiae (Sc; AF004731); C. elegans (Ce; O76258); S. carlbergensis pastorianus (Scp; Z86109). Leucines in heptad repeats are indicated with a star.

**B,** dendrogram of all TSG101 orthologues at the amino acid level. Phylogenetic analysis was performed using PHYLIP software and the majority-rule, strict-consensus tree program, creating an unrooted tree.

**C,** the domain structure of TSG101 orthologues and the closely related D. melanogaster protein CG9712. The inactive ubiquitin-conjugating domain (UbC), the proline-rich region (PRR), leucine zipper (LZ), and C-terminal helical (HX) domains are indicated.

**D,** helical wheel representation of the leucine zipper of mammalian TSG101. Each rotation of the helical wheel consists of seven amino acids, corresponding to the fit of seven amino acids into every two helical turns. The hydrophobic positions within the helical wheel are shaded.
hVPS28, perhaps indicating that the interaction with VPS28 plays a particularly important role in TSG101 function. Interestingly, the C-terminal helix is also conserved between TSG101 and other proteins possessing an inactive UbC domain (20).

**TSG101 and hVPS28 are Recruited to VPS4-induced Endosomes**—Previous data suggested that TSG101 regulates the transport of receptors to late endocytic compartments (15), but the mechanism by which this is achieved is unclear. TSG101 may interact directly with an endosome-associated receptor sorting machinery. Alternatively, given evidence that TSG101 is a transcriptional modulator (22, 23), it might elicit its effects indirectly by altering levels of expression of specific gene products. To provide evidence for a direct role for TSG101 and its interacting partner, hVPS28, in receptor sorting, we examined whether either could bind to endosomal membranes. Previous reports have shown that TSG101 is a predominantly cytosolic protein at steady state (49, 50), although it may also localize to the nucleus during S phase (49).

To demonstrate whether TSG101 and hVPS28 might bind at least transiently to endosomes, we looked for conditions that might stabilize their membrane association. Studies in yeast indicate that the endosome association of several class E vps proteins are coupled (4, 12). We therefore examined whether modulating the activity of another mammalian orthologue of a class E protein could affect the localization of TSG101 and hVPS28. Yeast Vps4p is a class E protein that is a member of the AAA family of proteins (5), many of which appear to participate in molecular rearrangement/unfolding reactions (51–53). On this basis it has been proposed that Vps4p acts to modulate interactions between other components of the vacuolar sorting pathway, including other class E proteins. Indeed, expression of Vps4p that is defective in ATP hydrolysis leads to the accumulation of Vps24p and Vps32p on the surface of aberrant endosomes in yeast (12), indicating that a deficiency in Vps4p function might prevent soluble components required for endocytic sorting from dissociating from the endosomal membrane. We have recently demonstrated that expression of ATPase-defective human VPS4 (hVPS4) gives rise to aberrant endosomes that are highly vacuolated and defective in sorting of cholesterol (10). Consistent with this, ATPase-defective mutants of the closely related mouse SKD1 prevent epidermal growth factor receptor degradation (11).

The localization of both TSG101 and hVPS28 were therefore examined in cells transfected with wild-type or ATPase-defective hVPS4, because expression of a dominant-negative mutant of hVPS4 might prevent the TSG101-hVPS28 complex from dissociating from aberrant endosomes. As demonstrated in Fig. 6A, TSG101 is largely cytosolic in untransfected cells, although the staining is somewhat granular. This distribution is similar to that observed for VPS28 (see Fig. 1G). Upon expression of ATPase-defective hVPS4, a portion of both TSG101 and hVPS28 became associated with aberrant, hVPS4-induced endosomal vacuoles (Fig. 6, B–E). Not all vacuoles were labeled with TSG101 or hVPS28, however, consistent with the heterogeneity of VPS4-induced structures observed in previous studies (10).

At present we do not know the mechanism underlying mutant VPS4-induced accumulation of TSG101-hVPS28 on the surface of endosomes. Although it is possible that VPS4 interacts directly with the TSG101 complex, it is more likely that a number of intervening components are present and that inhibition of VPS4 function causes a block in the normal cycling of all of these, causing a small portion of both TSG101 and hVPS28 to remain membrane-bound. How TSG101 associates with the endosome in the first place is also unclear. Intriguingly, however, the presence of a conserved UbC-like domain raises the possibility that TSG101 interacts with ubiquitinated component(s). There is increasing evidence that ubiquitination of receptors and their associated proteins plays a fundamental role in regulating the endocytic pathway (54), including post-endosomal sorting to the MVB and lysosome (55).

Our findings provide evidence that TSG101 and its interacting components associate with the endocytic pathway and therefore are likely to play a direct role in mitogenic receptor down-regulation, over and above any role they may play in gene regulation. It is important to stress that our data do not necessarily contradict previous reports concerning TSG101 function as a transcriptional regulator. Indeed, there is grow-

**Fig. 5. Identification of the hVPS28-interacting region of TSG101.** A, wild-type or the indicated domain deletions of TSG101-myc/His6 were translated in vitro with non-radioactive amino acids, and equal amounts were incubated with 35S-labeled, in vitro translated hVPS28. Samples were immunoprecipitated with anti-myc antibody and analyzed for 35S-hVPS28. The efficiency of translation of each TSG101-myc/His6 construct was verified by parallel translation reactions containing 35S-methionine. B, full-length (F), the leucine zipper plus helical (LZ + Hx), or the helical (Hx) domain alone of TSG101-myc/His6 were translated in vitro and treated as in A.

**Fig. 6. TSG101 and hVPS28 are recruited to VPS4-induced endosomes.** NRK cells were stained with anti-TSG101 antibody (A). Cells were transfected with eGFP-hVPS4(EQ) and visualized for eGFP (B, D), or stained for TSG101 (C) or VPS28 (E).
ing evidence of mechanistic links between the regulation of the endocytic pathway, nucleocytoplasmic communication, and gene regulation (see Ref. 56 for review (57)).

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