A Membrane Coat Complex Essential for Endosome-to-Golgi Retrograde Transport in Yeast

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Abstract. We have recently characterized three yeast gene products (Vps35p, Vps29p, and Vps30p) as candidate components of the sorting machinery required for the endosome-to-Golgi retrieval of the vacuolar protein sorting receptor Vps10p (Seaman, M.N.J., E.G. Marcusson, J.-L. Cereghino, and S.D. Emr. 1997. J. Cell Biol 137:79–92). By genetic and biochemical means we now show that Vps35p and Vps29p interact and form part of a multimeric membrane-associated complex that also contains Vps26p, Vps17p, and Vps5p. This complex, designated here as the retromer complex, assembles from two distinct subcomplexes comprising (a) Vps35p, Vps29p, and Vps26p; and (b) Vps5p and Vps17p. Density gradient fractionation of Golgi/endosomal/vesicular membranes reveals that Vps35p cofractionates with Vps5p/Vps17p in a vesicle-enriched dense membrane fraction. Furthermore, gel filtration analysis indicates that Vps35p and Vps5p are present on a population of vesicles and tubules slightly larger than COPI/coatomer-coated vesicles. We also show by immunogold EM that Vps5p is localized to discrete regions at the rims of the prevacuolar endosome where vesicles appear to be budding. Size fractionation of cytosolic and recombinant Vps5p reveals that Vps5p can self-assemble in vitro, suggesting that Vps5p may provide the mechanical impetus to drive vesicle formation. Based on these findings we propose a model in which Vps35p/Vps29p/Vps26p function to select cargo for retrieval, and Vps5p/Vps17p assemble onto the membrane to promote vesicle formation. Conservation of the yeast retromer complex components in higher eukaryotes suggests an important general role for this complex in endosome-to-Golgi retrieval.

Key words: retrieval • endosome • Golgi • vesicle • coat

The subcellular organelles that comprise the secretory and endocytic pathways perform the essential function of compartmentalizing often competing biochemical reactions. Each organelle contains a discrete set of resident proteins required for the proper functioning of that compartment. Vesicular carriers provide the vehicles to transport proteins and lipids between organelles, and thus are crucial for establishing and maintaining the distinct complement of proteins that define each organelle and determine its function (Rothman and Orci, 1992; Rothman and Wieland, 1996). Vesicle formation is mediated by cytoplasmic coat proteins that are recruited onto specific target membranes (Schekman and Orci, 1996). Presently the favored model for vesicle-mediated membrane traffic predicts that coat proteins perform two distinct functions: (a) cargo recognition and concentration; and (b) membrane deformation to generate a free vesicle. The vesicle coat sequesters a subset of membrane proteins by recognizing sorting determinants in the cytoplasmic tails of membrane proteins that are transported in vesicles (Kirchhausen et al., 1997). Finally, the vesicle coat must assemble into a structure capable of deforming the donor membrane into a small vesicle.

A prime example of the role of vesicular carriers in organelle maintenance is the transport of hydrolases to the lysosome. The lysosome is an acidic degradative organelle that contains many hydrolases required for protein, lipid, and carbohydrate turnover as well as downregulation of activated cell surface receptors through proteolytic degradation (Kornfeld and Mellman, 1989). Biosynthetic transport of hydrolases to the lysosome occurs in a receptor-mediated fashion. Hydrolases that have been modified in the Golgi complex by adding a mannose-6-phosphate moiety (M₆P)¹ are sorted away from proteins destined for se-

¹. Abbreviations used in this paper: CPY, carboxypeptidase Y; DPAP A, dipeptidyl amino peptidase A; DTSSP, 3,3’-dithiobis sulfosuccinimidyl-propionate; GST, glutathione S-transferase; HA, hemagglutinin; M₆P, mannose-6-phosphate.
cretion in the TGN by receptors that recognize M6P (Kornfeld, 1992). The ligand–receptor complexes are then packaged into vesicles for delivery to an endosomal compartment (Hille-Rehfeld, 1995; Traub and Kornfeld, 1997). The acidic lumen of the endosome triggers release of hydrolyses that are subsequently delivered to the lysosome. To maintain the forward flow of hydrolyses to the lysosome, the M6P receptor must be efficiently recycled back to the TGN and reused in additional rounds of hydrolyase sorting.

Sorting of the M6P receptor/ligand complexes into vesicles that bud from the TGN is believed to be mediated by the clathrin/AP-1 vesicle coat. The heterotetrameric AP-1 complex binds to the cytoplasmic tail of the M6P receptor via tyrosine-containing sequences (Glickman et al., 1989; Le Borgne and Hoflack, 1997; Le Borgne et al., 1993; Ohno et al., 1995). Clathrin then assembles onto the membrane-associated AP-1, and through its intrinsic self-assembly properties triggers formation of a budded vesicle (Pears and Robinson, 1990). Recycling the M6P receptor from the endosome to the TGN is also believed to be vesicle-mediated, and has been shown to require the small GTPase Rab9 (Riederer et al., 1994). However, the role of Rab9 in this transport pathway is believed to be downstream of vesicle formation, and currently there are no clear candidates for vesicle coat proteins acting in the retrieval of M6P receptors from the endosome to the Golgi.

This process is mirrored in the simple eukaryote Saccharomyces cerevisiae. In S. cerevisiae the vacuole is analogous to the mammalian lysosome. Genetic screens in yeast have identified more than 40 genes whose products are required for efficient delivery of vacuolar hydrolyses (Bankaitis et al., 1986; Robinson et al., 1988; Rothman and Stevens, 1986). These genes have been termed VPS for vacuolar protein sorting, and overlap partially with the PEP mutants isolated by screening for reduced protease activity (Jones, 1977). Phenotypic analysis of the vps mutants has classified the 40 or so complementation groups into six distinct classes (A–F) based upon criteria such as the morphological appearance of the vacuole and the severity of the defect in hydrolyase sorting (Banta et al., 1988; Raymond et al., 1992). Studies of the class A mutant vps10 revealed that the VPS10 gene encodes a type I transmembrane protein that functions as a sorting receptor for vacuolar hydrolyses, including carboxypeptidase Y (CPY; Marcusson et al., 1994; Westphal et al., 1996). Vps10p normally resides in a membrane fraction that also contains the late-Golgi enzyme Kex2p (Marcusson et al., 1994) and cycles between the Golgi and a prevacuolar endosome where it delivers CPY (and other hydrolyses) before being recycled back to the Golgi (Cereghino et al., 1995; Cooper and Stevens, 1996). Clathrin has also been shown to be important for sorting Vps10p (Seeger and Payne, 1992a) in the late-Golgi, and has been suggested to have a role both in sorting to the vacuole and also retention of late-Golgi membrane proteins such as the endopeptidase Kex2p (Payne and Schekman, 1989; Seeger and Payne, 1992b; Wilsbach and Payne, 1993b; Redding et al., 1996).

Recent detailed analysis of some of the class A (VPS29, VPS30 and VPS35) and class B (VPS5 and VPS17) vps mutants has shown that these genes are required for the proper localization of the Vps10 protein (Horazdovsky et al., 1997; Nothwehr and Hindes, 1997; Seaman et al., 1997). Disruption of VPS29, VPS30, or VPS35 resulted in almost complete mislocalization of the Vps10 receptor to the vacuole, causing CPY to be secreted from the cell. These gene products are also required for the proper localization of Kex2p and another Golgi-resident endopeptidase, dipeptidyl amino peptidase A (DPAP A; Seaman et al., 1997). As the VPS29, VPS30, or VPS35 gene products were not required for Golgi-to-endosome transport, we proposed that they may act in the retrieval/recycling of Vps10p from an endosomal compartment to the late-Golgi (Seaman et al., 1997).

VPS5 encodes a protein that is homologous to the mammalian SNX1 protein (Horazdovsky et al., 1997; Nothwehr and Hindes, 1997). SNX1 is involved in the endocytic trafficking of the epidermal growth factor receptor (EGFR), and was shown to interact with the tail of the EGFR (Kurten et al., 1996). An epitope-tagged Vps5p has been localized by immunofluorescence to an endosomal compartment (Nothwehr and Hindes, 1997), and the wild-type protein cofractionated with a dense pool of membranes (with a buoyant density similar to that of known coated vesicles) on Accudenz density gradients (Accurate Chemical & Scientific Corp., Westbury, NY; Horazdovsky et al., 1997). Vps5p and Vps17p form a stable complex, and have phenotypes indistinguishable from each other (Horazdovsky et al., 1997; Kohrer and Emr, 1993). In a vps5 mutant, greater than 50% of the Vps10p is mislocalized to a membrane fraction enriched in vacuolar markers (Horazdovsky et al., 1997).

Significantly, however, there are major differences between the phenotypes of the class A (vps29, 30 and 35) mutants and the class B (vps5 and 17) mutants (Banta et al., 1988; Raymond et al., 1992). First, in class B mutants the vacuole is fragmented into 10–20 smaller vacuoles, while class A mutants retain a vacuole with near wild-type morphology (Raymond et al., 1992). Class B mutants also exhibit a more severe defect in trafficking to the vacuole (Horazdovsky et al., 1997; Kohrer and Emr, 1993; Marcusson et al., 1994; Paravicini et al., 1992; Seaman et al., 1997). Hence, while the class A mutants have been proposed specifically to affect retrieval of a subset of membrane proteins such as Vps10p from the endosome to the Golgi, the class B genes have been proposed to act in a more general fashion such that when mutated, all endosome-to-Golgi recycling is abolished. This strong retrograde defect presumably also leads to a Golgi-to-endosome anterograde defect due to a lack of recycling of factors essential for forward transport. Thus, less Vps10p is mislocalized to the vacuole in a vps5 mutant than is observed for the class A vps29, vps30, and vps35 mutants.

Two other potential factors required for endosome to Golgi transport in yeast are the products of the VPS1 and VPS26 genes. vps1 and vps26 mutants are both classified as class F mutants with phenotypes intermediate to those of the class A and class B vps mutants (Raymond et al., 1992). VPS1 encodes a high molecular weight GTPase that belongs to the dynamin family (Nothwehr et al., 1995; Rothman et al., 1990). VPS1 function is required for the proper sorting of CPY to the vacuole (Nothwehr et al., 1995; Rothman et al., 1990; Vater et al., 1992), and is also important for the correct localization of Kex2p (Wilsbach and Payne, 1993a; Nothwehr et al., 1995). By analogy to
the established role of dynamin in the budding of clathrin-coated vesicles from the plasma membrane (Damen, 1996; Takei et al., 1996; Warnock and Schmid, 1996), it has been proposed that Vps1p acts with clathrin to sort CPY to the Golgi and possibly to maintain Kex2p in the late-Golgi (Wilsbach and Payne, 1993b). VPS26 is allelic to the PEP8 gene, and is also required for correct sorting of CPY to the vacuole (Bachhawat et al., 1994). Phenotypically, vps26 mutants are very similar to vps1 mutants, and thus it has been proposed that the two proteins function at the same point (Raymond et al., 1992). A recent screen undertaken specifically to identify mutants that fail to retain DPAP A in the late-Golgi resulted in the isolation of several mutants, many of which are allelic to the VPS genes described above (Nothwehr et al., 1996). For instance, the Golgi retention–defective (grd) mutant grd2 is allelic to vps5. grd4 is allelic to vps35, grd6 is allelic to vps26, and grd1 is allelic to vps1.

Here we show that Vps35p, Vps29p, Vps26p, Vps5p, and Vps17p associate with each other forming a multimeric complex, on vesicular and tubulovesicular membranes. The complex, which we call the retromer complex, appears to assemble onto the membrane from two distinct subcomplexes comprising (a) Vps35p, Vps29p, and Vps26p; and (b) Vps5p and Vps17p. Vps35p and Vps29p colocalize on sucrose density gradients in fractions with a density greater than Golgi and endosomal fractions. They are also found associated together on a population of vesicular membranes that separate away from Golgi membranes by gel filtration chromatography. Immunogold EM revealed that Vps35p is found in clusters on a prevacuolar endosome at sites where vesicles appear to be budding from the membrane. Both cytosolic and recombinant Vps5p can also assemble into a large complex, suggesting that it may have intrinsic self-assembly activity. In addition we have found that Vps35p assembles into a high molecular weight complex in the cytosol, and this assembly is dependent upon Vps29p. The phenotypic and biochemical data taken together suggest that these proteins function as a vesicle coat that directs the retrieval of the Vps10 receptor as well as other proteins from the endosome to the Golgi.

Materials and Methods

Reagents, Media, Yeast, and Bacterial Manipulations

Unless otherwise stated, reagents were obtained from Boehringer Mannheim Corp. (Indianapolis, IN), Fisher Scientific Co. (Fairlawn, NJ), or from Sigma Chemical Co. (St. Louis, MO). All yeast strains were grown in yeast extract, peptone, dextrose medium (YPD), or yeast nitrogen base medium (YNB; Sherman et al., 1979). Yeast transformations were performed using alkali cation treatment (Elble, 1992), and Escherichia coli transformations were performed according to Hanahan (1983). Yeast strains used in this study are listed in Table I.

Western Blotting

Samples solubilized in SDS-PAGE buffer (6 M urea, 50 mM Tris-HCl pH 6.8, 1% SDS) were loaded and electrophoresed (constant current of 20 mA per gel) on either 8% or 10% polyacrylamide gels. After electrophoresis, the gel was briefly immersed in transfer buffer (5% methanol, 50 mM Tris, 380 mM glycine) and then placed flat against a sheet of nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) and assembled into an apparatus (Bio-Rad Laboratories, Hercules, CA) for electrophoretic transfer of the proteins onto the nitrocellulose. Transfer was accomplished at constant current (150 mA) for 10–12 h. The blot was then blocked with blocking buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.25% gelatin, 0.1% Nonidet P40, 0.1% sodium azide) for an hour before incubation with antibodies. Most antiserum was used at a dilution of 1:1,000. Antibody incubations were generally for 2 h at room temperature on a rocking table. Washes were for 30–60 min with several changes of blocking buffer. [125I]protein A (Amersham Corp., Arlington Heights, IL) was used to detect the first antibodies, and this was used at a dilution of 1:1,000. After further blocking, the blots were exposed to x-ray film (Eastman Kodak Co., Rochester, NY).

CPY Sorting Assays and Subcellular Fractionation Procedures

CPY sorting was performed as described previously in Seaman et al. (1997). Similarly, cell fractionation experiments using differential centrifugation to separate P100 membranes from P13 membranes and cytosol (S100) were performed essentially as described previously in Seaman et al. (1997). Sucrose density gradient fractionation of P100 membranes was performed as in Seaman et al. (1997) with the exception that a P100 membrane fraction was loaded onto the gradient instead of a supernatant from a 13,000 g spin. The P100 generated for this experiment was from the lysis of ~15 ODs of cells, and was resuspended in 0.5 ml of lysis buffer and briefly dounced in a 1 ml dounce homogenizer before loading onto the sucrose density gradient. Fractionation of P100 membranes by sephacryl S1000 column chromatography was performed as follows. Cells grown in YPD media were harvested at an OD of 2–3 (generally 6,000–7,000 ODs of cells were used). The cells were spun down using an RC5B centrifuge (Sorvall, Newtown, CT) in a GS3 rotor (10 min at 5,000 rpm). The cell pellet was washed with water and spun again. The cells were then resuspended into 500 ml of spheroplasting buffer (0.8 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl2, 2 mM DTT, and 10 μg/OD zymolyase) and incubated at 30°C with gentle shaking (90 rpm). Spheroplasting was monitored by measuring the OD 600 nm of an aliquot of cells placed in 1 ml of water. When spheroplasting was 80–90% complete (~60 min), the cells were spun out again (this time in a GSA rotor) for 10 min at 4°C and 1,500 rpm. The cell pellet was washed with wash buffer (0.8 M sorbitol, 20 mM Hepes-KOH, pH 7.0, 50 mM potassium acetate, 2 mM EDTA) and then pelleted again as before. The cells were then lysed in chilled lysis buffer (0.2 M sorbitol, 20 mM Hepes-KOH pH 7.0, 50 mM potassium acetate, 2 mM EDTA) plus protease inhibitors (PMSF, 100 μg/ml, pepstatin 1 μg/ml, leupeptin 1 μg/ml, antipain 5 μg/ml and o2-macrooglobin 10 μg/ml) with the aid of a 15-m1 dounce homogenizer (~15 strokes). The lysate was spun at 13,000 g for 10 min in a Sorval SS34 rotor (10,500 rpm) at 4°C. The resulting supernatant was centrifuged for 1 h at 100,000 g in a 60 ti rotor (32,000 rpm; Beckman Instruments, Inc., Fullerton, CA) at 4°C. The resulting P100 pellet was re-suspended in ~1.5 ml of lysis buffer with the aid of a 1 ml dounce homogenizer, was briefly spun at 13,000 g to pellet any aggregated material, and was then loaded onto a sephacryl S1000 (Pharmacia, Piscataway, NJ) column (118 cm x 1.5 cm) that had been equilibrated with lysis buffer. The column was run at 4°C, the samples were eluted using a flow rate of ~250 μl per min. and 2.5-ml fractions were collected. Fractions were analyzed.
for various proteins by Western blotting, and were also analyzed by EM. To strip the P100 membranes, lysis buffer containing 250 mM NaCl was used to suspend the membranes at the 100,000-g spin. The membranes were dounced in a 1-ml dounce as described previously, and were then centrifuged again at 100,000 g to pellet the stripped membranes. The supernatant was then fractionated on the sephacryl S300 column as described below. Alternatively, the supernatant was dialyzed against ~1-liter lysis buffer (0.2 M sorbitol, 20 mM Heps-KOH, pH 7.0, 50 mM potassium acetate, 2 mM EDTA) overnight at 4°C. After dialysis, the supernatant was spun briefly at 13,000 g, and was then fractionated on a sephacryl S300 column.

**Cross-linking of Membrane Fractions or Yeast Lysates**

Cells grown in YNB media were first spheroplasted and then labeled for various proteins by Western blotting, and were also analyzed by EM. Cells grown in YNB media were first spheroplasted and then labeled for various proteins by Western blotting, and were also analyzed by EM.

**Fractionation of Yeast Cytosol**

Yeast cells grown in YPD media were harvested at an OD 600 nm of ~1.0. Approximately 200 ODs of cells were spun down in a bench-top clinical centrifuge. The pellet was washed with water and then resuspended in 50 mM potassium acetate, 2 mM EDTA overnight at 4°C. After dialysis, the supernatant was spun briefly at 13,000 g, and was then fractionated on a sephacryl S300 column.

**Plasmid Construction, Generation of the vps35dn Allele, and Cloning and Disruption of VPS26**

Standard molecular biology techniques and protocols for DNA manipulation were used throughout this study (Sambrook et al., 1989). To generate an antisera against Vps29p, a glutathione S-transferase (GST) fusion (Smith and Johnson, 1988) with Vps29p was created. Using an oligonucleotide primer incorporating a BamHI site at the 5’ end and a primer corresponding to the T3 promoter in the region of VPS29, the intron (Seaman et al., 1997) was amplified by PCR from pVPS29-1 (Seaman et al., 1997). The ~1.6-kb fragment was cloned using the TA cloning vector according to manufacturer’s instructions (Invitrogen Corp., Carlsbad, CA). The fragment was excised by BamHI digestion, and was subcloned into pGEX 3X (Smith and Johnson, 1988) that had been digested with BamHI to generate pGST-VPS29. E. coli transformed with pGST-VPS29 was grown in liquid culture and induced to express isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The protein produced was isolated by glutathione affinity chromatography (Smith and Johnson, 1988) and following the manufacturer’s instructions, and on SDS gels was found to migrate as a protein with an apparent size of ~120 kD on SDS polyacrylamide gels. This was much larger than the predicted size of the fusion (~50 kD), and may have been due to the very acidic nature of the Vps29 protein, which has a pl of ~4.3. Purified fusion protein was used to immunize New Zealand white rabbits following a standard immunization protocol (Harlow and Lane, 1988). After four boosts, the animal was bled out, and 5 ml of antisera was affinity-purified on GST-Vps29p that had been coupled to CNBr activated sepharose (Pharmacia Biotech, Inc.) following the manufacturer’s instructions.

The vps35dn allele was generated and screened for in an identical manner to the vps35ts allele (Seaman et al., 1997) with the exception that the gapped plasmid was a multi-copy plasmid (pRS424) containing VPS35. VPS26 was cloned by amplifying the gene using PCR and genomic DNA as template. The primers used for the PCR reaction were designed to flank the open reading frame, annealing ~200 bp on either side of the VPS26 gene. A single product of ~1.7 kb was obtained from the PCR, and was cloned using the TA cloning kit to create pTA26. VPS26 was then subcloned into Nor1, and Kpn1 digested pRS414 and pRS416 after excising VPS26 from pTA26 by Not1 and Kpn1 digestion. The resulting plasmids (pVPS26-414 and pVPS26-416) were transformed into SEY26-1, a yeast strain carrying a mutation in VPS26. Both plasmids were able to complement the CPY sorting defect in SEY26-1 (data not shown). A deletion of VPS26 was generated by removing ~80% of the coding region of VPS26 by digesting pVPS26-416 with HindIII and Xba1. A HindIII-XbaI fragment containing the LEU2 gene excised from pBlueScript (Stratagene, La Jolla, CA) containing LEU2 was then subcloned into the cut pVPS26-416 and ligated. The resulting plasmid pVPS26-LEU2 was used as a template to amplify the LEU2 gene using 33-mer primers as used before. The PCR product was used to transform wild-type yeast (SEY6210). Transformants were selected by growth on -leu plates. Deletion of the gene was confirmed by PCR on genomic DNA prepared from leu+ transformants. Transformation of the vps26a strain (MSY2600) with pVPS26-416 or pVPS26-416 was able to complement the CPY sorting defect observed in strain MSY2600. An epitope-tagged version of VPS26 that complements a vps26a mutant has been reported previously (Bachhawat et al., 1994), and was a gift from E. Jones (Carnegie-Mellon University, Pittsburgh, PA). However, it was necessary to subclone a HindIII fragment from the integrating plasmid phHA22 (Bachhawat et al., 1994) into HindIII-cut pVP26-416 to generate an expression plasmid for the hemagglutinin (HA)-tagged VPS26. This subcloning created pVPS26-HA, which was transformed into MSY2600 and was found to complement the CPY sorting defect fully.

A yeast strain with a myc-tagged VPS10 gene integrated into the genome was generated in the following way. The plasmid containing VPS10- myc, pEMY10-21 (Cereghino et al., 1995), was cut with BglII to excise the VPS10-myc construct. The linear DNA was gel-purified, and was used to transform the strain EMY3 (vps10A::HIS3) to which was transformed with pCY10 (Bankaitis et al., 1986) that encodes a CPY-Invertase fusion. Transformants were selected for growth on -ura plates. Replicas were screened for the ability to sort CPY-Invertase to the vacuole using a plate assay (Paravicini et al., 1992). Cells in which the VPS10-myc had integrated into the genome scored as white colonies capable of sorting CPY-Invertase to the vacuole. Four independent colonies were found to be VPS+. These colonies also contain the VPS10-myc gene as determined by the presence of the ~200-kD band in immunoprecipitates from 32P-labeled cells using the anti-myc antibody 9E10 (data not shown). Three of the four were also found to now be his+, indicating that the VPS10-myc...
gene had integrated into the vps10:HI33 locus, displacing the HI33 gene in the process. One of the his− VPS+ colonies was designated MSY10-21, and was used in these studies.

To generate significant amounts of Vps5p in bacteria for in vitro studies, a GST-VPS5 fusion was generated. This was achieved by subcloning an XbaI-XhoI fragment of VPS5 into XbaI-XhoI digested pGEX KG (Pharmacia Biotech., Inc.). The resulting plasmid was termed pGST-VPS5. Digestion at the XbaI site in Vps5p resulted in the loss of six amino-terminal residues of Vps5p and XhoI cut downstream of the coding region. This procedure formed an in-frame fusion of VPS5 with GST, which expressed almost full-length VPS5. An identical fusion was made between VPS5 and GST in Mrug, a GST expression system for yeast (Mitchell et al., 1993). This plasmid was designated pMSGST-5, and when expressed in vps5Δ, cells were able to complement the CPY sorting defect fully (data not shown).

Bacteria harboring pGST-VPS5 were induced to express by adding IPTG. The fusion protein was soluble, although poorly expressed. A protein of ~120 kD was observed on SDS polyacrylamide gels, which corresponded to the full-length fusion. Some proteolysis of the GST-VPS5 occurred in the bacteria, resulting in breakdown products ranging in size from 60 to 30 kD. GST-Vps5p that had been eluted from the glutathione-sepharose was dialyzed against 50% of the CPY being secreted from the cell as the Golgi-hydrolase receptor, Vps10p. Strains carrying mutations in the VPS35, VPS29, or VPS30 genes have phenotypes that are virtually indistinguishable, and cause Vps10p to become mislocalized to the vacuolar membrane (Seaman et al., 1997). Detailed analysis of the vps35 mutant strongly suggested that a defect in the retrieval of Vps10p from the prevacuolar endosome to the late-Golgi was responsible for the vacuolar protein-sorting defect (Seaman et al., 1997). The very similar phenotypes of the vps35, vps29, and vps30 mutants suggested that the gene products may all function at a common step in the retrieval pathway. To test this directly and to gain a better understanding of the roles that the VPS35, VPS29, and VPS30 genes play in the retrieval of Vps10p, a dominant negative mutant of VPS35 (vps35dn) was generated. When introduced at high copy number into wild-type cells, this allele caused a significant defect in the trafficking of CPY to the vacuole, resulting in 50% of the CPY being secreted from the cell as the Golgi-

**Affinity Isolation of GST-Vps5p from Yeast Lysates**

Wild-type and vps5a cells harboring a plasmid to express GST-Vps5p in yeast (pMSGST-5) were grown in -ura -leu minimal media to an OD 600 nm of ~1 OD unit/mL. 1.00 OD 600 nm equivalents of cells were harvested and converted to spheroplasts by zymolyase digestion. The spheroplasted cells were lysed at 100 OD 600 nm equivalents per ml in lysis buffer (0.2 M sorbitol, 20 mM Hepes-KOH, pH 7.0, 50 mM potassium acetate, 2 mM EDTA) containing 0.5% triton with the aid of a dounce homogenizer. The lysate was spun at 20,000 g in a Sorvall SS34 rotor for 10 min at 4°C to remove insoluble material. The supernatant was collected and incubated with 150 μl of glutathione sepharose slurry for 15 min at room temperature on a rocking table. After several washes of the sepharose with 10-ml aliquots of lysis buffer, the bound proteins were eluted using 10 mM of reduced glutathione in 50 mM Tris, pH 8.0. The proteins were precipitated by adding TCA to 10%, and after acetone washes were resuspended into SDS-PAGE sample buffer.

**Electron Microscopy**

EM on frozen yeast cells or frozen P100 membrane fractions to localize Vps5p was performed essentially as described previously (Rieder et al., 1996; McCaffery and Farquhar, 1995). vps5Δ cells (SEY 4-1) transformed with the plasmid pAH31 to express an HA-tagged Vps5p (Nothwehr and Hindes, 1997) were grown to midlog (OD 600 nm 0.6) before being spun down and fixed for 18–24 h in 3% formaldehyde dissolved in PBS, pH 7.4. The cells were then dispersed in 1% agarose, which was trimmed into mm3 blocks and then cryoprotected in 2.3 M sucrose containing 25% polyvinylpyrrolidone (10,000 D) and frozen onto cryopins in liquid nitrogen. Ultrathin cryosections were cut on a Reichert Ultracut E (Leica, Deerfield, IL) equipped with an FC4 cryostage, and were collected onto 300 mesh, formvar/carbon-coated grids and immunolabeled as described previously (Reider et al., 1996). Sections were adsorption stained/embedded with 3.2% polyvinyl alcohol, 0.2% methyl cellulose (400 centiposes), and 0.1% uranyl acetate contained in H2O. Monoclonal anti-HA antibodies (Berkeley Antibody Co., Inc., Richmond, CA) were used to label the HA-tagged Vps5p, and these were visualized using anti-mouse coupled to 5 nm colloidal-gold.

For size-fractionated Vps5p complexes, the complexes were adsorbed to 300 mesh, formvar/carbon-coated grids (freshly iron) fixed for 10 min in 3% formaldehyde dissolved in PBS, pH 7.4, and were subsequently immunolabeled (not as) described previously (McCaffery and Farquhar, 1995). The complexes were then negatively stained in 2% uranyl acetate and observed on a JEOL 1200 EX II TEM in paraformaldehyde as in Rieder et al. (1996).

**Results**

**Vps35p and Vps29p Interact**

Previous studies have identified several genes whose products are required for the correct trafficking of the vacuolar...
modified p2 precursor form (Fig. 1A, lanes 1 and 2). Effects of dominant negative alleles are often due to titration of other factors by the dominant negative protein. Candidates for these other factors in this case would clearly include Vps29p and/or Vps30p. Therefore, the strain carrying the vps35dn allele was transformed with high-copy plasmids to overexpress either the VPS29, VPS30, or VPS35 gene products. Only cells overexpressing VPS29 were able to suppress the CPY sorting defect caused by the vps35dn allele (Fig. 1A, lanes 3 and 4). These data suggest an interaction between Vps35p and Vps29p.

To address whether this genetic interaction corresponded to a physical interaction between Vps35p and Vps29p, we first investigated the subcellular localization of Vps29p. If Vps35p and Vps29p physically interact, then it is logical to expect the proteins to colocalize. To investigate the subcellular localization of Vps29p, a polyclonal antiserum was raised against a recombinant GST-Vps29p fusion protein (Materials and Methods). This antiserum recognized a protein with an apparent molecular mass of 45 that was absent in immunoprecipitations from vps29Δ cells, and was greatly elevated in cells containing a multicopy VPS29 plasmid (Fig. 1B and data not shown). The apparent molecular mass of Vps29p is significantly larger than its predicted molecular mass of ~30. This discrepancy may be due to some form of posttranslational modification, or could be caused by the acidic nature of Vps29p, which has a pI of ~4.3. Some cross-reactivity of the antiserum with a protein(s) slightly larger than Vps29p was observed, and is seen in Fig. 1B, panel 2 when a lysate from vps29Δ cells was treated with the Vps29p antiserum. When the subcellular distribution of Vps29p was investigated (Fig. 1B, panel 1) it was found to be a predominantly soluble protein, but ~20% of Vps29p was associated with the 100,000 g particulate fraction (P100) that contains Vps35p (Paravicini et al., 1992) and the Golgi membrane proteins Kex2p and Vps10p (Marcussen et al., 1994). No Vps29p was detected in the P13 fraction that contains vacuolar membranes, ER, mitochondria, and plasma membranes (Marcussen et al., 1994). Deletion of VPS35 resulted in Vps29p becoming completely soluble (Fig. 1B, panel 4), demonstrating that Vps35p is required for membrane association of Vps29p. In contrast, there was no change in the localization of Vps29p in cells in which VPS30 was deleted (Fig. 1B, panel 3).

**Vps35p and Vps29p Form a Multimeric Complex with Vps5p and Vps17p**

To investigate further the potential association of Vps35p with Vps29p, and to identify any other proteins that may interact with the Vps35p/Vps29p complex, a series of cross-linking experiments was undertaken. Lysates from wild-type cells that had been cleared of unbroken cells were treated with the homobifunctional reducible cross-linker DTSSP. The reaction was stopped by adding TCA and the sample was denatured in a buffer containing SDS and urea to break noncovalent interactions. Vps35p or Vps29p was immunoprecipitated from resulting lysates under nonreducing conditions. As seen in Fig. 2A, when no cross-linker is added, the antisera against Vps35p only immunoprecipitated Vps35p (lane 1). Similarly, the Vps29p antisera predominately rec-

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**Figure 2.** Vps35p and Vps29p are part of a multimeric membrane associated complex. (A) A cleared lysate from [35S]methionine-labeled wild-type cells (SEY 6210) was either left untreated (lanes 1 and 2) or treated with cross-linker (lanes 3 and 4) as described in Materials and Methods. After cross-linking, the proteins were precipitated, and either Vps35p (lanes 1 and 3) or Vps29p (lanes 2 and 4) was immunoprecipitated. The immunoprecipitates were subjected to SDS-PAGE on an 8% gel, and the proteins were visualized by fluorography. Strikingly, the pattern of bands seen in the cross-linked samples (lanes 3 and 4) was very similar, indicating that Vps35p and Vps29p interact with a common set of proteins. (B) P100 membranes were isolated from wild-type (SEY 6210) [35S]methionine-labeled cells and resuspended into 100 μl of lysis buffer. The cross-linker DTSSP was added, and the samples were allowed to cross-link for 30 min at room temperature. After cross-linking, precipitation by adding TCA, and resuspending the proteins, Vps35p (lanes 1, 2, 4, 6, and 7) or Vps29p (lane 3) was immunoprecipitated from the lysate. These primary immunoprecipitates were then resuspended into 100 μl of lysis buffer, and in the case of lanes 2, 4, 6, and 7, reducing agent was added. 900 μl of immunoprecipitation buffer was added, and the samples were further immunoprecipitated with antibodies against either Vps35p (lane 1), Vps29p (lanes 2, 3, and 4), Vps30p (lane 6), or Vps5 (lane 7). The supernatant from the sample in lane 1 (after the primary immunoprecipitation) was retained, and antibodies against Vps5p were added (lane 5). Proteins were resolved on an 8% SDS polyacrylamide gel. Vps35p could be cross-linked to Vps29p, and the proteins designated as p95 and p70 were found to have an identical migration to Vps5p and Vps17p. Immunoprecipitation of Vps5p from cross-linked lysates demonstrated the presence of this protein in a complex that also contained Vps35p and Vps29p. (C) GST-Vps5p expressed in yeast interacts with Vps17p, Vps35p, and Vps29p. Vps35Δ cells (BYH152) expressing either pMSGST-5 or pEG (KT, empty vector) (Mitchell et al., 1993) were lysed in Heps lysis buffer containing 0.5% Triton. The lysate was cleared by centrifugation and then incubated with glutathione-sepharose for 15 min. After several washes, the bound proteins were eluted and precipitated. The proteins were resolubilized in SDS-PAGE sample buffer, separated on an 8% polyacrylamide gel, and then transferred to nitrocellulose for Western blotting. GST-Vps5p was able to associate with Vps17p, Vps35p, and Vps29p (lane 2). GST alone did not associate with Vps17p, Vps35p, or Vps29p (lane 1).
ognized Vps29p (lane 2). When cross-linker was added and either Vps35p (lane 3) or Vps29p (lane 4) was immuno-precipitated, a number of other proteins were detected. Vps29p cross-linked to a protein with identical mobility to Vps35p, and both Vps29p and Vps35p cross-linked to three other proteins—p95, p70, and p50—based upon their respective migrations on the SDS polyacrylamide gel.

A faint band corresponding to Vps29p was also present in the Vps35p immunoprecipitates. The relatively low Vps29p signal in the Vps35p immunoprecipitates is due to two factors: first, the cross-linking reaction is not completely efficient, which means that not all Vps29p associated with Vps35p will be cross-linked; and second, Vps29p is a small protein with relatively few methionines and cysteines, and hence will not give a strong signal compared with larger proteins, such as Vps35p.

The interaction between Vps35p and Vps29p indeed was confirmed in an experiment in which an enriched source of Vps35p was used as a source of material for cross-linking. 80% of the total Vps35p is present in an endosome/Golgi/vesicle-enriched fraction that sediments at 100,000 g (P100). Therefore, P100 membranes isolated by centrifugation from [35S]methionine-labeled wild-type cells were resuspended in lysis buffer and then treated with cross-linker. Vps35p or Vps29p was immunoprecipitated from the lysate using specific antisera. In the presence of cross-linker, Vps35p or Vps29p was immunoprecipitated with larger proteins, such as Vps35p.

As an independent test of the interactions discovered through the use of cross-linkers, a fusion protein between GST and Vps5p was expressed in yeast, and was used to confirm the presence of the multimeric complex containing Vps35p, Vps29p, Vps17p, and Vps5p. The GST-Vps5p was functional, as expression in a vps5Δ strain was able to complement the CPY sorting defect fully (data not shown). GST-Vps5p isolated from either wild-type (data not shown) or vps5Δ (Fig. 2 C) yeast lysates using glutathione-sepharose was found to be bound to Vps17p, Vps35p, and Vps29p (Fig. 2 C, lane 2), but none of these proteins was detected when a control lysate from a strain expressing just GST was treated with glutathione-sepharose (Fig. 2 C, lane 1).

**Vps26p is the p50 Component of the Multimeric Complex**

To identify the p50 component of the multimeric complex, a database search of the cloned VPS and PEP genes was performed to identify candidate 50-kD proteins. The best candidate was VPS26/PEP8. This gene encodes an ~40-kD protein that migrates on SDS gels with a molecular mass of ~50 (Bachhawat et al., 1994). The phenotype of vps26 mutants is intermediate between that of the class A mutants (vps35, vps29, and vps30) and the class B mutants (vps5 and vps17) (Raymond et al., 1992). Therefore, a strain in which VPS26 was deleted was generated as described in Materials and Methods, and was used in a cross-linking experiment to determine if Vps26p is p50. In Fig. 3 A, lysates were treated with cross-linker as before, and Vps35p was immunoprecipitated. When no cross-linker was added, only Vps35p was present (lane 1), while in lane 2, cross-linker was added to a lysate, and the pattern of bands contained Vps5p, Vps17p, p50, and Vps29p. However, when an identical experiment was performed using lysate from cells in which VPS26 was deleted, the p50 band was missing (compare lanes 2 and 3). It is interesting to note that Vps35p continues to coimmunoprecipitate with Vps5p, Vps17p, and Vps29p, suggesting that Vps26p is not essential for formation of the complex. More recently, cross-linking experiments using a strain expressing an epitope-tagged Vps26p have confirmed that Vps26p does indeed interact with Vps35p, Vps29p, Vps5p, and Vps17p in a large complex (data not shown).

**Assembly of the Multimeric Complex Requires both Vps29p and Vps35p**

To address the question of which components of the complex are essential for forming the Vps35p/Vps29p/Vps26p/Vps5p/Vps17p complex, lysates from various [35S]methionine-labeled yeast strains were treated with cross-linker, and either Vps35p or Vps29p was immunoprecipitated. Because the localization of the proteins is affected in the various strains tested (e.g., Vps35p is localized to the P13, vacuole-enriched fraction in a vps29Δ strain), a crude lysate...
Vps26p is required for the correct localization of Vps10p

VPS26/PEP8 has been shown previously to be important for sorting CPY to the vacuole (Bachhawat et al., 1994). The data presented here suggested that Vps26p is part of a multimeric membrane-associated complex that contains Vps35p, Vps29p, Vps5p, and Vps17p. If Vps26p is indeed interacting with Vps35p/Vps29p and/or Vps5p/Vps17p, then it is reasonable to expect a vps26 mutant to have a similar phenotype(s) to either a vps35 or vps5 mutant. Therefore, cell fractionation experiments were performed in which localization of Vps10p was investigated in both wild-type and vps26Δ strains. It has been observed that Vps10p is mislocalized in both vps5 and vps35 mutants (Horazdovsky et al., 1997; Seaman et al., 1997), although to a lesser extent in vps5 mutants. In Fig. 4 the distribution of Vps10p was examined using a differential centrifugation method that separates the smaller Golgi, endosomal, and vesicular membranes away from larger vacuolar (and also ER, mitochondrial, and plasma) membranes (Marcusson et al., 1994). Wild-type cells exhibited a normal distribution of Vps10p with ∼90% of the Vps10p residing in a P100 membrane fraction that contains Golgi, endosomal, and vesicular membranes. In vps26Δ cells ∼90% of Vps10p is found in the vacuole-enriched P13. This means that vps26Δ cells, with respect to the localization of Vps10p, have a phenotype very similar to the vps29 or vps35 mutants (Seaman et al., 1997). Interestingly, a significant proportion of Vps35p becomes soluble in vps26Δ cells (data not shown), which makes vps26 mutants somewhat unique, as other mutants (e.g., vps29 or vps5) do not affect the membrane association of Vps35p, but rather cause Vps35p to shift to a different population of membranes (Seaman et al., 1997 and data not shown).

Figure 4. Vps26p is required for the correct localization of Vps10p.

Vps26p is required for the correct localization of Vps10p. Wild-type (SEY 6210) or vps26Δ (MSY2600) mutant cells were converted to spheroplasts and labeled with [35S]methionine for 15 min. Excess cold methionine/cysteine was then added, and the cells were chased for 45 min, after which the cells were lysed in Hepes buffer and the lysate was spun at 2,000 rpm to remove unbroken cells. Differential centrifugation (13,000 g, then 100,000 g) was then used to separate larger membranes such as vacuoles (P13) from small membrane compartments such as Golgi, endosomes, vesicles (P100), and cytosol (S100). The fractions were treated with TCA to precipitate the proteins that were then washed with acetone and resuspended into immunoprecipitation buffer. Vps10p was recovered from the different fractions by immunoprecipitation, and was resolved by SDS-PAGE. The proteins were visualized by fluorography. Deletion of VPS26 causes a dramatic shift in the distribution of Vps10p to a vacuolar membrane fraction.
Vps35p and Vps5p Colocalize on Sucrose Density Gradients

Previous studies have shown that the distribution of Vps35p on sucrose gradients can be resolved into two distinct populations of membranes (Seaman et al., 1997), while Vps5p has been shown to cofractionate with membranes of a density similar to those of small vesicles such as COPI-coated vesicles (Horazdovsky et al., 1997). The cross-linking data suggest that Vps35p and Vps5p should be present on the same population of membranes. To test this hypothesis, a P100 membrane fraction prepared from [35S]methionine-labeled cells was spun to equilibrium on a sucrose density gradient. Fractions were collected, and various proteins were immunoprecipitated to determine their location in the gradient. Consistent with previously published data, Vps35p is found distributed between two populations of membranes (Fig. 5). Furthermore, there is clear colocalization between Vps35p and Vps5p in the denser fractions (fractions 8 and 9). Based upon the fractionation profiles of the late-Golgi protein Kex2p and the endosomal t-SNARE Pep12p (Becherer et al., 1996), it is possible to suggest that the lighter population of membranes which Vps35p associates (fractions 5 and 6) is most likely an endosomal compartment. The fractions in which Vps35p and Vps5p colocalize (fractions 8 and 9) appear to be more dense than the fractions that contain Kex2p, and could correspond to a population of coated vesicles or tubules.

![Figure 5. Colocalization of Vps35p and Vps5p in a dense membrane fraction.](image)

Vps35p and Vps5p are Present in Vesicle-enriched Membrane Fractions

The data presented here and previously (Horazdovsky et al., 1997; Seaman et al., 1997) suggest that Vps5p and Vps35p are associated with a dense population of membranes whose buoyant density is similar to those of known coated vesicles. As most vesicles also have a characteristic size in the range of 40–80 nm (Schekman and Orci, 1996), we have investigated the size of the membranes on which Vps35p and Vps5p are associated by gel filtration chromatography. A yeast strain expressing an epitope-tagged Vps10p was fractionated to generate a P100 membrane fraction, which was then loaded onto a sephacryl S1000 column. The fractions collected were assayed for the presence of various proteins by Western blotting. We observed that Vps10p was most abundant in fractions 60–66 (Fig. 6A), where the late-Golgi protein Kex2p (fractions 60–62) was also detected (data not shown). The bulk of Vps35p and Vps5p coeluted slightly later in fractions 66–72. These fractions also contained Vps29p (data not shown), indicating that Vps35p and Vps5p are present on membranes somewhat smaller than the membranes that contain the bulk of Vps10p (i.e., Golgi and endosomal membranes). As a marker for a known coated vesicle, the distribution of...
Two Distinct Vps Subcomplexes are Present in a 100,000 g Membrane Fraction

How do the proteins (Vps35p, Vps29p, Vps26p, Vps17p, and Vps5p) assemble onto the membrane, and what is the nature of the membrane-associated complex? To address this question, we have attempted to strip the complex off membranes and then separate the components of the complex by gel filtration. Previous studies have shown that Vps35p and Vps5p can be stripped off the membrane by treatment with 1M NaCl (Horazdovsky et al., 1997; Paravicini et al., 1992), and in our preliminary experiments we found that as little as 250 mM NaCl would also strip Vps35p and Vps5p from the membrane (data not shown). Therefore, a P100 membrane fraction was treated with a buffer containing 250 mM NaCl to strip the membranes. The soluble proteins were then separated away from the stripped membranes by centrifugation, and the supernatant was loaded onto a sephacryl S300 column that had been equilibrated in buffer containing 250 mM NaCl. Fractons from the column were assayed for components of the multimeric complex by Western blotting.

We observed that Vps5p coeluted with Vps17p (Fig. 8), suggesting that they remain associated. Some proteolysis of the Vps5 protein occurs during this procedure, resulting in two breakdown products that migrate a little below the intact Vps5p. Vps5p and Vps17p coeluted with a predicted size of ~400–500 kD, which is significantly larger than a simple dimer of Vps5p/Vps17p, which has a combined molecular mass of ~140 kD. Similarly, Vps35p coeluted with Vps29p, consistent with the data from the cross-linking ex-

Figure 7. Immunolocalization of Vps5p by EM. vps4 cells (SEY 4-1) harboring the plasmid pAH31 (HA-tagged VPS5) (Nothwehr and Hindes, 1997) were fixed and prepared for cryoelectron microscopy. (A) Frozen thin sections were labeled with the anti-HA antibody to localize Vps5p, and were followed by 5 nm anti-mouse colloidal-gold. Labeling was found to be restricted to discrete regions of the prevacuolar endosomal membrane (Ec), and appeared most concentrated at the swollen rim of the cisterna (B). Also shown are the nucleus (n) and plasma membrane (pm). Bar, 100 nm.
Vps5p Exhibits Self-assembly Properties

So far this report has focused primarily on the membrane-associated pool of the proteins being described. It must be noted, however, that a significant proportion of each of the proteins is also found in the cytoplasm (Table II). For example, ~40–60% of the cellular Vps5p is soluble (Horazdovsky et al., 1997; Nothwehr and Hindes, 1997). The only exception is Vps35p, of which only 5% of the total Vps35p is present in the cytoplasm (Paravicini et al., 1992; Seaman et al., 1997). It is reasonable to suppose that at least a part of the function of these proteins involves cycling on and off the membrane. We may therefore gain some insight into how these proteins assemble on the membrane by studying their properties in the cytosol. Thus, cytosol was prepared from wild-type cells, and was subjected to sephacryl S300 column chromatography. Fractions collected were assayed for various proteins by Western blotting. In Fig. 9 A the graph depicts the elution profile of Vps5p, which eluted in the void volume and appeared to be part of a very large (>10^6 D) complex. This profile contrasts with the data presented in Fig. 8, in which Vps5p that had been stripped off the membrane with NaCl was found to fractionate with a predicted size of ~400–500 kD. However, that fractionation was performed in the presence of 250 mM NaCl, which may have disrupted protein–protein interactions. Therefore, to see if Vps5p will assemble into the >10^6 D complex in the absence of NaCl, a P100 membrane fraction was stripped with NaCl as before. The membranes were spun out, and the soluble proteins were dialyzed against Heps lysis buffer to remove the salt gradually. This dialyzed supernatant was then fractionated on the sephacryl S300 column. In Fig. 9 B, dialyzed Vps5p was found in the void volume fraction, indicating that it can assemble into a >10^6-D complex in the absence of salt. Interestingly, it was found that fractionation of cytosolic Vps5p was unchanged in any mutant tested, including a vps17Δ mutant (data not shown), hinting that Vps5p alone may possess this self-assembly activity.

When the fractionation of Vps35p and Vps29p was investigated, it was found that they too coeluted in the void volume fraction, and thus could be part of a large cytosolic complex (Fig. 9, C and D, solid lines). It must be noted, however, that the bulk of Vps29p was found in fractions consistent with its predicted molecular weight. In vps29Δ cells, the bulk of the cytosolic Vps35p was no longer in the void volume fraction, but was shifted to a later fraction (Fig. 9 C, broken line). Similarly, in the absence of Vps35p, cytosolic Vps29p no longer eluted in the void volume fraction (Fig. 9 D, broken line). Thus, Vps35p and Vps29p associate with each other in the cytoplasm in a complex that is >10^6 D.

Recombinant Vps5p Displays Self-assembly Activity

The experiments performed on both cytosolic Vps5p and Vps5p that had been stripped from membranes and then allowed to reassemble into a large complex (Fig. 9, A and B) suggested that Vps5p may possess intrinsic self-assembly activity. This was most strongly suggested when cytosol from vps17Δ cells was fractionated. Even though Vps5p stably associates with Vps17p (Horazdovsky et al., 1997), cytosolic Vps5p from vps17Δ cells continued to elute in the void volume fraction (data not shown), suggesting a self-assembly property intrinsic to Vps5p. This hypothesis was tested directly by expression of Vps5p as a recombinant fusion protein. A GST-Vps5p fusion expressed in yeast has previously been shown to be functional (Fig. 2 C), and hence an identical fusion was expressed in bacteria. The GST-Vps5p was purified by glutathione affinity

Table II. Subcellular Distribution of Components of the Multimeric Complex

| Protein | P13 | S100 | P100 | Reference               |
|---------|-----|------|------|-------------------------|
| Vps35p  | 15% | 5%   | 80%  | Paravicini et al., 1992 |
| Vps29p  | 0%  | 80%  | 20%  | This study              |
| Vps26p  | 0%  | 0–30%| 70–100%| Bachhawat et al., 1994 |
| Vps5p   | 0%  | ~50% | ~50% | Horazdovsky et al., 1997|
| Vps17p  | 0%  | ~25% | ~75% | Kohrer and Emr, 1993   |

Figure 8. Sizing the components of the multimeric complex reveals the existence of two distinct subcomplexes. P100 membranes prepared from wild-type cells (SEY 6210) were stripped by resuspending the pellet in Heps lysis buffer containing 250 mM NaCl. The membranes were pelleted by centrifugation at 100,000 g. The supernatant was loaded onto a sephacryl S300 column equilibrated with lysis buffer plus 250 mM NaCl. Fractions were precipitated with TCA and analyzed by Western blotting. The bulk of Vps5p and Vps17p remain associated with each other and form a complex with a predicted size of 400–500 kD. Vps35p remains associated with Vps29p in an ~230-kD complex.
chromatography, dialyzed against Hepes lysis buffer, and fractionated on a sephacryl S300 column. In Fig. 10 A, the elution profile from the column is shown. GST-Vps5p (solid line) eluted in the void volume fraction, while GST alone (broken line) eluted in fractions consistent with its molecular weight. Fractions from the gel filtration column were analyzed by SDS-PAGE (Fig. 10 B). Lane M contains molecular weight markers, and lane L contains a sample of the material loaded onto the column. It is apparent that GST-Vps5p is very susceptible to degradation, and that most of the protein loaded onto the column is in fact a breakdown product of ~55 kD. However, the majority of the protein that elutes in the void volume fraction is full-length GST-Vps5p with a molecular mass of ~120 kD. As the breakdown products of the GST-Vps5p were not enriched in the void volume fraction, it seems unlikely that the assembly of GST-Vps5p into the high molecular weight complex was not the result of nonspecific aggregation.

Furthermore, analysis of the void volume fraction by EM revealed that the GST-Vps5p had assembled into a complex of ~10^6 D. In C, the fractionation of cytosolic Vps35p is analyzed. Vps35p is found in the void volume fraction in cytosol prepared from wild-type cells (SEY 6210) (solid line). However, when cytosol is prepared from vps29Δ cells (PSY 1-29), the bulk of Vps35p now elutes in a later fraction (broken line), indicating that Vps29p function is required for Vps35p to assemble into a large complex. Similarly in D, Vps29p in wild-type (SEY 6210) cytosol will partially fractionate in the void volume fraction (solid line), but when cytosol is prepared from vps35Δ cells (EMY 18), all the detectable Vps29p is found in later fractions. Thus, Vps35p and Vps29p appear to assemble together into a large complex that elutes in the void volume from a sephacryl S300 column.
Previous studies have identified a number of endosome-to-Golgi retrieval mutants. The first group comprises vps35, vps29, and vps30, all of which cause a severe defect in trafficking of CPY to the vacuole, even though another vacuolar hydrolase, proteinase A (PrA), reaches the vacuole and is matured with wild-type kinetics (Seaman et al., 1997). The defect in the trafficking of CPY to the vacuole is the result of complete mislocalization of Vps10p to the vacuole membrane in the vps35, vps29, and vps30 mutants. These mutants therefore appear to affect localization of a subset of proteins, one of which is Vps10p, and have been proposed to act in a cargo-selective manner in the endosome-to-Golgi retrieval pathway (Seaman et al., 1997).

The second group comprises vps5 and vps17 mutants. Superficially, the vps5 and vps17 mutants have quite different phenotypes from the first group. In vps5 and vps17 mutants there is a significant defect in PrA maturation, and the vacuoles appear fragmented. Vps10p is mislocalized to the vacuolar membrane in vps5 and vps17 mutants; however, this mislocalization is less dramatic than that observed in the vps35, vps29, and vps30 mutants. Thus, it was suggested that Vps5p and Vps17p are part of a more general mechanism for endosome-to-Golgi retrieval such that the vps5 and vps17 mutants cause a complete block in retrieval, ultimately leading to a partial defect in forward transport from the Golgi to the endosome due to lack of retrieval of factors required for forward transport (Horazdovsky et al., 1997).

Here we show that five peripheral membrane proteins—Vps35p, Vps29p, Vps26p, Vps5p, and Vps17p—assemble together to form a multimeric complex on a prevacuolar membrane. Vps5p has been localized to the prevacuolar membrane, and was found to be clustered at specific sites that appear to be nascent budding vesicles. Furthermore, Vps5p can assemble in vitro in a large macromolecular structure. These data, along with previous studies and phenotypic characterization of the various mutants, point to a role for these proteins as a membrane coat complex that directs retrieval of Vps10p and other molecules from the endosome to the Golgi.

Assembly of the Multimeric Retromer Complex from Two Distinct Subcomplexes

The behavior and properties of the individual components of the retromer complex have been examined to understand the role that each component plays within the complex. A genetic interaction between VPS35 and VPS29 was identified through the use of a dominant negative allele of VPS35. Only overexpression of VPS29 was able to suppress the CPY sorting defect seen in cells carrying the vps35dn allele. This result could be interpreted two ways: either (a) the dominant negative vps35 titrated Vps29p out of the system into a nonfunctional complex; the resulting loss of Vps29p would then cause the defect in CPY sorting; or (b) Vps29p acts downstream of Vps35p, and hence

Discussion

A New Membrane Coat Complex, the Retromer for Endosome-to-Golgi Retrieval

Previous studies have identified a number of vps mutants that affect the localization of the vacuolar hydrolase receptor Vps10p, and have been proposed to act in the endosome-to-Golgi retrieval of not only Vps10p, but other late-Golgi resident proteins such as Kex2p and DPAP A (Horazdovsky et al., 1997; Nothwehr and Hindes, 1997; Seaman et al., 1997). These mutants could be divided into two distinct groups based upon phenotypic analysis. The first group comprises vps35, vps29, and vps30, all of which
overexpression can bypass the defect observed in cells overexpressing the vps35Dh allele. We favor the former, as no suppression of the previously described (Seaman et al., 1997) temperature conditional allele of vps35 has been observed in cells overexpressing Vps29p (Seaman and Emr, unpublished data), which would be expected if Vps29p acts downstream of Vps35p. Furthermore, cell fractionation experiments clearly show that the proportion of Vps29p that associates with a P100 (Golgi/endosome/vesicle) membrane fraction requires the presence of Vps35p for that membrane association.

Cross-linking of both a crude lysate and the P100 membrane fraction demonstrated a direct interaction between Vps35p and Vps29p, and also revealed the presence of three other proteins in a complex with Vps35p/Vps29p. These proteins were subsequently identified as Vps26p, Vps5p, and Vps17p, and are all peripheral membrane proteins that are found in both the cytoplasm and on P100 membranes (Table II). Cross-linking studies performed on extracts of mutant cells indicated that the retromer complex in fact comprises two distinct subcomplexes. When lysates from vps5 or vps17 mutant cells were cross-linked, it was found that Vps35p remained associated with both Vps29p and Vps26p. As previous studies had reported that Vps5p and Vps17p form a stable complex with each other (Horazdovsky et al., 1997), the two retromer subcomplexes would then comprise Vps35p/Vps29p/Vps26p and Vps5p/Vps17p. These data were supported by an experiment in which P100 membranes were stripped with salt to dissociate the peripheral membrane proteins. When the solubilized proteins were subjected to gel filtration to size the individual components, it was observed that Vps35p remained associated with Vps17p in a complex with a predicted size of ~400–500 kD. This value is significantly larger than the sum of the molecular masses of the individual components (~140 kD), and requires further investigation to establish the nature of this complex. One possibility, however, is that Vps35p/Vps17p can assemble into structures containing three or possibly four copies of a simple Vps5p/Vps17p dimer, producing a multimer of ~400–500 kD. Although larger than its predicted size, the Vps35p/Vps29p complex (~230 kD) may also contain Vps26p (~40 kD). Association of Vps26p with the Vps35p/Vps29p complex remains to be proven, however, and awaits production of antisera against endogenous Vps26p. Mutants of vps26 cause Vps10p to become localized to a vacuolar membrane fraction in a manner indistinguishable from that of vps35 or vps29 mutants, consistent with Vps26p interacting with Vps35p and/or Vps29p. Furthermore, the acute instability of Vps35p in a double vps29Δ/vps26Δ mutant supports the notion that Vps35p forms a subcomplex with both Vps29p and Vps26p. Although vps30 mutants have phenotypes almost identical to those of vps29 and vps35 mutants, no Vps30p was detected in the retromer complex, and the cross-linking data indicated that Vps30p was not required for complex formation. Although untested, it seems likely that Vps30p may somehow act to regulate complex formation, or alternatively Vps30p may function downstream of the retromer complex.

It is worth noting that the components of this cross-linkable membrane-associated retromer complex display varying degrees of membrane association. Vps35p is 80% membrane-associated, while only 20% of Vps29p is localized to the membrane, and Vps5p/Vps17p and Vps26p fall in between these two values (Table II). If all five proteins were stably associated with each other all of the time, it would be reasonable to expect them all to be equally membrane-associated or cytosolic, which is clearly not the case. Hence, it seems likely that formation and disassembly of the complex is a dynamic process involving cycling on and off the membrane.

**The Self-assembly Properties Intrinsic to Vps5p Suggest a Mechanical Role in Vesicle Formation**

As all the proteins that comprise the retromer complex are peripheral membrane proteins, and therefore presumably cycle between the cytoplasm and the membrane, cytosol was size-fractionated to investigate what sort of complexes exist in the cytoplasm. Strikingly, it was found that cytosolic Vps5p existed in a very large complex that eluted in the void volume fraction with a predicted size >10^6 D. As this fractionation was performed in a buffer that did not contain high salt, it seemed possible that in the absence of the salt, Vps5p could assemble (presumably with other proteins; for example, Vps17p) into a large complex. This possibility was tested directly by allowing the retromer complex components that had been salt-stripped from membranes to assemble in vitro by dialyzing away the salt. It was found that Vps5p could indeed assemble into a large complex that was >10^6 D. Even though Vps5p forms a stable complex with Vps17p and requires Vps17p for its membrane association (Horazdovsky et al., 1997), Vps17p was not required for Vps5p to assemble into the >10^6-D complex. Interestingly, however, we have found that Vps17p in vps5Δ cells is very unstable, and is rapidly degraded with a half-life of ~30 min (Seaman and Emr, unpublished data). These data hint that in vivo, Vps5p and Vps17p play different roles within their subcomplex. Vps17p is required for membrane association, while Vps5p appears to have intrinsic assembly activity. To test the ability of Vps5p to assemble in vitro, a fusion protein expressed in bacteria was purified by affinity chromatography. Consistent with the behavior of cytosolic Vps5p, the recombinant GST-Vps5p was found to assemble into a complex that elutes in the void volume from a gel filtration column, and forms homogenous particles 15–20 nm in diameter. These structures, however, probably do not perfectly mirror the structures that endogenous Vps5p forms in vivo, as Vps17p would be expected to play a role in determining how and where Vps5p assembles in the cell.

It appeared from the gel filtration analysis of cytosol that Vps35p could also assemble into a large structure. This activity was dependent upon Vps29p, and may reflect an oligomerization property of Vps35p. As almost all of the Vps35p in the cell is membrane-associated (Paravicini et al., 1992), this in vitro assembly of cytosolic Vps35p may reflect an in vivo property of the membrane-associated Vps35p. We can imagine that if Vps35p acts to recruit specific cargo (e.g., Vps10p) into vesicles for retrieval, a clustering of Vps35p through some sort of oligomerization would facilitate the concentration of receptors in a specific membrane domain. Vps29p would then serve a catalytic
role, activating Vps35p and promoting its oligomerization. Thus, in a vps29 mutant, Vps35p may remain associated with the membrane, and may still associate with the cytoplasmic tails of receptors such as Vps10p, but will no longer cluster to concentrate the receptors into a vesicle. This hypothesis is consistent with the observed behavior of Vps35p in a vps29 mutant, in which Vps35p follows Vps10p to a vacuolar membrane fraction (Seaman et al., 1997).

**Localization of Vps5p In Vivo Suggests a Role in Vesicle Budding**

Sucrose density gradient analysis of the P100 membrane population with which Vps35p and Vps5p are associated suggested that Vps35p is associated with two distinct pools of membranes: a lighter endosomal fraction and a denser membrane fraction. Vps5p is predominantly associated with only the denser pool of membranes, which has previously been shown to have a buoyant density very similar to known coat vesicles such as COPI and COPI (Horazdovsky et al., 1997). These observations are consistent with the hypothesis that Vps35p/Vps29p (and also Vps26p) are involved in the recruitment of specific cargo into vesicles. A proportion of Vps35p and Vps29p would logically be expected to be associated with endosomal membranes, and not present in vesicles. Vps5p/Vps17p may then assemble onto the membrane where the receptors are clustered, and drive the budding of a vesicle. Size fractionation of membranes by sephacryl S1000 column chromatography shows that Vps5p and Vps35p colfractionate on a population of membranes smaller than Golgi membranes where Vps10p and Kex2p reside, but slightly larger than the COPI-coated vesicles.

When fractions from the sephacryl S1000 column were analyzed by EM, they were found to contain numerous small 40–100-nm vesicles. However, attempts to label these fractions with antibodies against retromer components were unsuccessful, possibly because the membrane association of the retromer proteins is somewhat labile, and during the course of fractionation or possibly after elution from the column, the proteins dissociated from the membrane. Therefore, a cruder P100 membrane fraction was labeled with antibodies against an epitope-tagged VPS5, and was found to be predominantly localized to vesicular and tubulovesicular structures. In vivo localization of Vps5p was achieved using a class E mutant (vps4) that accumulates an exaggerated endosomal compartment (Babst et al., 1997; Rieder et al., 1996). In this instance, Vps5p was found to be localized to the swollen rims of the cisternal membranes that are characteristic of the class E endosome. These structures are not dissimilar to the membranes with which the epitope-tagged Vps5p is associated when a P100 membrane fraction is analyzed by cryoimmunogold EM. The key observation is that Vps5p is clustered on specific regions of the prevacuolar compartment, not evenly distributed throughout as has been shown for the vacuolar ATPase that also accumulates in the prevacuolar endosome in a class E mutant (Rieder et al., 1996). That Vps5p appears localized to the swollen rims of the cisternae is consistent with its proposed role in vesicle formation.

**Conservation of the Retromer Components and their Role in Receptor Trafficking**

Mammalian homologues of the VPS35, VPS29, VPS26, and VPS5 genes have been identified either as ESTs, or as full-length cDNA clones. VPS35 is homologous to the mouse gene Mem3 (Hwang et al., 1996), VPS29 has EST homologues in both mouse and humans (Seaman et al., 1997), and the mammalian homologue of VPS26/PEP8 (Bachhawat et al., 1994) is termed Hb38, and is essential for embryogenesis in the mouse (Lee et al., 1992). Interestingly, Vps26p shares modest sequence homology (27% identity over a stretch of 130 aas) with the Sec21 protein, a component of the yeast COPII vesicle coat. The best-characterized mammalian homologue of the retromer components is SNX1, which is homologous to VPS5 (Horazdovsky et al., 1997; Nothwehr and Hindes, 1997). SNX1 has been shown to interact with the cytoplasmic tail of the EGF receptor, and overexpression of SNX1 causes an increase in the turnover and degradation of the EGF receptor (Kurten et al., 1996). It has been suggested that SNX1 is required for endosome-to-lysosome trafficking, and may play a role in the downregulation of activated receptor tyrosine kinases (Kurten et al., 1996). SNX1 and Vps5p share most sequence homology in a conserved domain that has been termed the PX domain (Ponting, 1996). The precise role that this conserved domain plays in the function of Vps5p and SNX1 remains to be determined, but it is interesting to note that Vps17p also contains a PX domain, as does Grd19p (Voos and Stevens, 1998) and Mvp1p (Ekena and Stevens, 1995), two proteins both believed to be important for endosome-to-Golgi retrieval in yeast. The PX domain may therefore confer a function common to all these proteins, perhaps targeting to an endosomal membrane by binding a specific lipid. This mechanism, however, remains to be tested.

**A Model for the Mechanism of Retromer-mediated Endosome-to-Golgi Retrieval**

The data presented in this report are consistent with the complex containing Vps35p, Vps29p, Vps26p, Vps5p, and Vps17p acting in formation of vesicles for endosome-to-Golgi retrieval, but do not exclude the possibility that other proteins also function in this pathway (e.g., Grd19p, Mvp1p, Vps30p, and Vps1p). One of these candidates (Vps1p, a dynamin-like GTPase) has been shown to be required for proper localization of Kex2p (Wilsbach and Payne, 1993a). Furthermore, vps1 and vps26 mutants have been classified in the same phenotypic group, implying an interaction or action of both proteins at the same step (Raymond et al., 1992).

Clathrin has also been proposed to act in retrieval from the endosome to the Golgi, and clathrin mutants also affect the localization of Kex2p (Payne and Schekman, 1989; Seeger and Payne, 1992b; Wilsbach and Payne, 1993b). Interestingly, however, the effect of clathrin inactivation upon the localization of Kex2p occurs independently of the putative late-Golgi localization signal in the cytoplasmic tail of Kex2p (Redding et al., 1996). Hence, it seems more likely that clathrin functions at the Golgi, and not in endosome-to-Golgi retrieval.
Thus, we propose that Vps35p acts with Vps29p and Vps26p to select cargo for retrieval from the endosome to the Golgi by interactions (either directly or indirectly via accessory factors) with the cytoplasmic tails of proteins such as Vps10p and Kex2p (Fig. 10). Aromatic amino acid-containing motifs in the tails of Vps10p and Kex2p have been shown to be important for their retrieval to the Golgi (Schweizer et al., 1997). Vps5p/Vps17p assemble onto the clustered receptors through interactions with Vps35p/Vps29p/Vps26p and/or the membrane. The self-assembly activity of Vps5p may drive the budding of the vesicle, although this function may also be provided by a different component of the retromer complex or by an as yet unidentified protein. The retromer complex therefore appears to display the two key activities associated with a vesicle coat complex–cargo recognition and self-assembly. Further studies are required to elucidate the precise role the retromer components play in endosome-to-Golgi retrieval, and the regulatory mechanisms such as a docking protein and/or a small GTPase that govern vesicle coat assembly on endosomal membranes have also yet to be revealed. However, these proteins provide the best candidate for a vesicle coat complex functioning in endosome-to-Golgi retrieval, and a similar complex may function in mammalian cells at an analogous point. Cloning and characterization of the mammalian homologues of the yeast retromer components will aid the molecular dissection of endosome-to-Golgi retrieval.

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