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-

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; DPAP A, dipeptidyl amino peptidase A; DTSSP, 3,3′-dithiobis sulfosuccinimidyl-proprionate; 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 hydrolases that are subsequently delivered to the lysosome. To maintain the forward flow of hydrolases to the lysosome, the M6P receptor must be efficiently recycled back to the TGN and reused in additional rounds of hydrolase 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 (Pearse 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 identifed more than 40 genes whose products are required for efficient delivery of vacuolar hydrolases (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 hydrolase 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 hydrolases, 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 hydrolases) 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, 1992*a*) 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, 1992*b*; 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, 1993*a*; Nothwehr et al., 1995). By analogy to

the established role of dynamin in the budding of clathrin-coated vesicles from the plasma membrane (Damke, 1996; Takei et al., 1996; Warnock and Schmid, 1996), it has been proposed that Vps1p acts with clathrin to sort CPY at the Golgi and possibly to maintain Kex2p in the late-Golgi (Wilsbach and Payne, 1993*b*). *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*. *grd9* 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 Vps5p 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 Vps5p 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.

Table I. Yeast Stains Used in This Study

Strain	Genotype	Source
SEY 6210	*MATα leu2-3, 112 ura3-52 his 3Δ200 trp1-Δ901 lys 2-801 suc2-Δ9*	Robinson et al., 1988
EMY18	SEY 6210 *vps35Δ::HIS3*	Seaman et al., 1997
EMY3	SEY 6210 *vps10Δ::HIS3*	Marcusson et al., 1994
PSY1-29	SEY 6210 *vps29Δ::HIS3*	Seaman et al., 1997
JCY3000	SEY 6210 *vps30Δ::HIS3*	Seaman et al., 1997
SEY 26-1	SEY 6210 *vps26-1*	Robinson et al., 1988
MSY2600	SEY6210 *vps26Δ::LEU2*	This study
KKY11	BHY11 *vps17Δ::HIS3*	Kohrer and Emr, 1993
BHY152	SEY 6210 *vps5Δ::HIS3*	Horazdovsky et al., 1997
MSY10-21	SEY 6210 *VPS10::VPS10-myc*	This study
SEY 4-1	SEY 6210 *vps4-1*	Robinson et al., 1988

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, 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. [^{125}I]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 was 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 $CaCl_2$, 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, 100μg/ml, pepstatin 1 μg/ml, leupeptin 1 μg/ml, antipain 5 μg/ml and α2-macroglobulin 10 μg/ml) with the aid of a 15-ml 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 resuspended 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 × 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 resuspend the membranes after 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 Hepes-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 15 min with [^{35}S]methionine/cysteine. Excess cold methionine and cysteine were added, and the cells were chased for 45 min. The cells were then spun out and resuspended in lysis buffer (20 mM Hepes-KOH, pH 7.0, 50 mM potassium acetate, 2 mM EDTA, 0.2 M sorbitol) at ~7 ODs/ml. A lysate was produced by douncing the cells in a dounce homogenizer using 15 strokes of the pestle. The lysate was then transferred to eppendorf tubes and spun at 300 *g* for 5 min at 4°C to remove unbroken cells and large organelles such as nuclei. In some cases the resulting cleared lysate was then treated with the cross-linker 3,3′-dithiobis sulfosuccinimidyl-proprionate (DTSSP; Pierce Chemical Co., Rockford, IL), which was added to a final concentration of 2 mM. In other experiments, the cleared lysate was first spun at 13,000 *g* for 5 min at 4°C to remove vacuolar membranes, mitochondria, plasma membranes, and endoplasmic reticulum. The supernatant from this spin was centrifuged further by spinning at 100,000 *g* for 1 h to generate a P100. This supernatant was then resuspended into 100 μl of lysis buffer with the aid of a plastic pestle, and cross-linker was added to a final concentration of 2 mM. Cross-linking was allowed to proceed for 30 min at room temperature. The reaction was stopped by adding TCA to a final concentration of 10%, and the tubes were placed on ice for 20 min to allow the proteins to precipitate. The precipitated proteins were pelleted by centrifugation at 13,000 *g* for 5 min, and were then washed twice with ice-cold acetone to remove the TCA. The samples were lyophilized and then resuspended into 100 μl of urea buffer (6 M urea, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS). Protein pellets were resuspended with the aid of a sonicating waterbath. 1 ml of immunoprecipitation buffer was added (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA), the samples were spun for 10 min at 13,000 *g* to remove insoluble debris, and then antisera was added. Immunoprecipitations were carried out as described previously (Seaman et al., 1997).

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 ml of spheroplasting buffer (0.8 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$). DTT and zymolyase (Seikagaku, Tokyo, Japan) were added to final concentrations of 2 mM and 10 μg/OD, respectively. Spheroplasting was monitored by observing the drop in OD 600 nm when an aliquot of the spheroplasting cells was placed in water. When spheroplasting was 80–90% complete, the cells were spun out as before and washed with chilled wash buffer (0.8 M sorbitol, 20 mM Hepes-KOH, pH 7.0, 50 mM potassium acetate, 2 mM EDTA). After recovering the cells by centrifugation, the cells were resuspended into lysis buffer at ~100 ODs per ml. The cells were lysed with the aid of a dounce homogenizer. The lysate was spun at 13,000 *g* at 4°C to remove unbroken cells and larger membranes. The supernatant was then spun for 1 h at 4°C and 100,000 *g* to pellet the remaining membranes. 1 ml of the supernatant (yeast cytosol) was loaded onto a sephacryl S300 (Pharmacia Biotech, Inc., Piscataway, NJ) column that had been equilibrated with lysis buffer, and was then fractionated with a flow rate of 0.4 ml per min using lysis buffer to elute the proteins. 1.25-ml fractions were collected after an initial wait period of 80 min. The fractions were precipitated with 10% TCA, and the pellets were washed with acetone to remove the TCA as described before. The proteins were resuspended into 100 μl of urea sample buffer (6 M urea, 50 mM Tris-HCl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue). 30 μl of the sample was loaded per lane onto an 8% SDS polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose for Western blotting.

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 BamH1 site at the 5′ end and a primer corresponding to the T3 promoter, the region of *VPS29* downstream of the intron (Seaman et al., 1997) was amplified by PCR from p*VPS29 -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 BamH1 digestion, and was subcloned into pGEX 3X (Smith and Johnson, 1988) that had been digested with BamH1 to generate pGST-VPS29. *E. coli* transformed with pGST-VPS29 was grown in liquid culture and induced to express by adding 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 by 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 pI 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 multicopy 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 *Not*1, and *Kpn*1 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 compliment 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 disrupted gene by PCR. The same primers were used as 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 *vps26*Δ strain (MSY2600) with pVPS26-414 or pVPS26-416 was able to compliment the CPY sorting defect observed in strain MSY2600 (data not shown). An epitope-tagged version of *VPS26* that compliments a *vps26* 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 p8HA22 (Bachhawat et al., 1994) into HindIII-cut pVPS26-416 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 compliment 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 (*vps10*Δ::*HIS3*; Marcusson et al., 1994) along with pCYI50 (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 contained the *VPS10-myc* gene as determined by the presence of the ~200-kD band in immunoprecipitates from ^{35}S-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*::*HIS3* locus, displacing the *HIS3* 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 *Xba*1-*Xho*1 fragment of *VPS5* into *Xba*1-*Xho*1 digested pGEX KG (Pharmacia Biotech., Inc.). The resulting plasmid was termed p*GST-VPS5*. Digestion at the Xba1 site in Vps5p resulted in the loss of six amino terminal residues of Vps5p and Xho1 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 pEG KT, a GST expression system for yeast (Mitchell et al., 1993). This plasmid was designated pMSGST-5, and when expressed in *vps5*Δ, cells were able to compliment 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-Vps5p 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 ~1 liter of lysis buffer (0.2 M sorbitol, 20 mM Hepes-KOH, pH 7.0, 50 mM potassium acetate, 2 mM EDTA) overnight at 4°C. This buffer was loaded onto the sephacryl S300 column and fractionated in a manner identical to that for yeast cytosol.

Affinity Isolation of GST-Vps5p from Yeast Lysates

Wild-type and *vps5*Δ 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 ODU/mL. 1,000 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). *vps4* 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 mm^3 blocks and then cryoprotected in 2.3 M sucrose containing 25% polyvinylpyrollidone (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 H_2O. 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 ionized) fixed for 10 min in 3% formaldehyde dissolved in PBS, pH 7.4, and were subsequently immunolabeled (or 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 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-

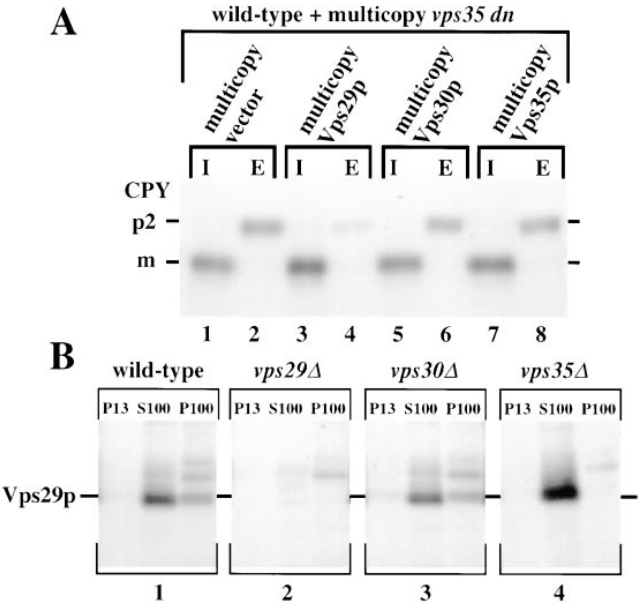

Figure 1. Vps35p functionally interacts with Vps29p. (*A*) Wild-type (SEY 6210) cells expressing a dominant negative *vps35* allele were transformed with multicopy (2μ) plasmids to overexpress *VPS29*, *VPS30,* or *VPS35*. Control cells were transformed with empty vector. Cells were pulse-labeled with [^{35}S]methionine for 10 min, and were then chased in the presence of excess cold methionine and cysteine for 30 min. The cells were then converted to spheroplasts, and intracellular and extracellular fractions were separated by centrifugation. CPY was immunoprecipitated from resulting lysates, and was subjected to electrophoresis on an 8% SDS polyacrylamide gel. The proteins were visualized by fluorography. (*B*) Wild-type (SEY 6210) and mutant strains in which *VPS29*, *VPS30,* or *VPS35* are deleted (PSY1-29, JCY3000, and EMY18, respectively) were converted to spheroplasts and then labeled for 15 min with [^{35}S]methionine. After adding excess cold methionine/cysteine, the cells were chased for 45 min to allow the labeled proteins to reach a steady state distribution. The cells were then lysed and fractionated by differential centrifugation. Vps29p was immunoprecipitated from the resulting fractions, and was then resolved on an 8% SDS polyacrylamide gel and visualized by fluorography. The association of Vps29p with a 100,000 *g* membrane fraction (P100) was found to be dependent upon Vps35p.

modified p2 precursor form (Fig. 1 *A*, 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. 1 *A*, 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 *vps29*Δ cells, and was greatly elevated in cells containing a multicopy *VPS29* plasmid (Fig. 1 *B* 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. 1 *B,* panel *2* when a lysate from *vps29*Δ cells was treated with the Vps29p antiserum. When the subcellular distribution of Vps29p was investigated (Fig. 1 *B,* 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 (Marcusson et al., 1994). No Vps29p was detected in the P13 fraction that contains vacuolar membranes, ER, mitochondria, and plasma membranes (Marcusson et al., 1994). Deletion of *VPS35* resulted in Vps29p becoming completely soluble (Fig. 1 *B*, 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. 1 *B*, 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 to precipitate the proteins, and then 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. 2 *A,* when no cross-linker is added, the antisera against Vps35p only immunoprecipitated Vps35p (lane *1*). Similarly, the Vps29p antisera predominately rec-

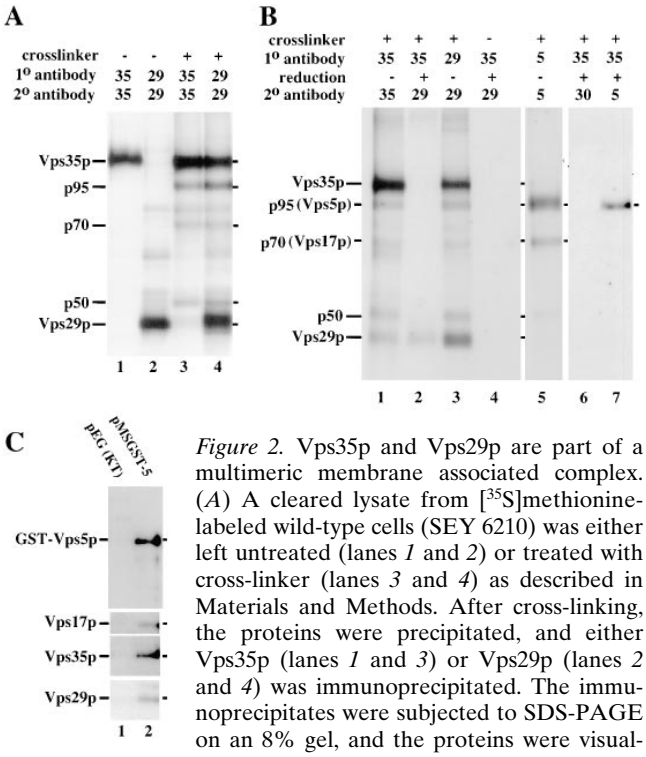

Figure 2. Vps35p and Vps29p are part of a multimeric membrane associated complex. (*A*) A cleared lysate from [^{35}S]methionine-labeled wild-type cells (SEY 6210) was either left untreated (lanes *1* and *2*) or treated with cross-linker (lanes *3* and *4*) as described in Materials and Methods. After 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) [^{35}S]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 urea 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. *vps5*Δ cells (BHY152) expressing either pMSGST-5 or pEG (*KT*; *empty vector*) (Mitchell et al., 1993) were lysed in Hepes 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 immunoprecipitated, 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 [^{35}S]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 (Fig. 2 *B*, lane *1*), Vps35p coimmunoprecipitates with Vps29p and the three other proteins: p95, p70, and p50. The identity of Vps29p was confirmed by treating cross-linked immunoprecipitated Vps35p with reducing agent to break the cross-linker. Vps29p is then immunoprecipitated from the resulting lysate (lane *2*). In lane *3*, antibodies against Vps29p immunoprecipitated both Vps29p and Vps35p, along with the three other proteins that coimmunoprecipitate with Vps35p (compare lanes *1* and *3*). The association of Vps29p with Vps35p was cross-linker–dependent; when no cross-linker was added to the P100 membranes, Vps29p did not coimmunoprecipitate with Vps35p (lane *4*). As a control for efficient cross-linking, Vps5p was immunoprecipitated from the extracts used for lane *1* (lane *5*). Vps5p has been shown previously to be stably associated with Vps17p (Horazdovsky et al., 1997), and hence this interaction was deemed to be a suitable positive control for cross-linking. It was noticed that Vps5p and Vps17p exhibited mobilities very similar if not identical to two of the proteins (p95 and p70) that coimmunoprecipitated with Vps35p and Vps29p. Therefore, to test directly the possibility that Vps5p and Vps17p are interacting with Vps35p/Vps29p, P100 membranes were cross-linked as before, and Vps35p was immunoprecipitated from the resulting lysates. After treating the immunoprecipitates with reducing agent to break the cross-linker, antibodies against Vps30p, Vps5p, or Vps17p were added. It was not possible to detect Vps30p after reducing the Vps35p immunoprecipitate (lane *6*); however, antiserum against Vps5p did immunoprecipitate a protein with the mobility of Vps5p from the reduced lysate (lane *7*), and Vps17p could also be detected, although to a lesser extent (data not shown).

Previously published data have demonstrated that the P100 membrane–associated Vps35p can be floated out of 60% sucrose using a simple two-step sucrose gradient (Paravicini et al., 1992). Vps5p has been found to behave in a similar fashion (Horazdovsky et al., 1997) and therefore the cross-linked complex described here is unlikely to represent a very large sedimentable particle, but rather a peripheral membrane-associated complex.

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 *vps5*Δ strain was able to complement the CPY sorting defect fully (data not shown). GST-Vps5p isolated from either wild-type (data not shown) or *vps5*Δ (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 [^{35}S]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 *vps29*Δ strain), a crude lysate

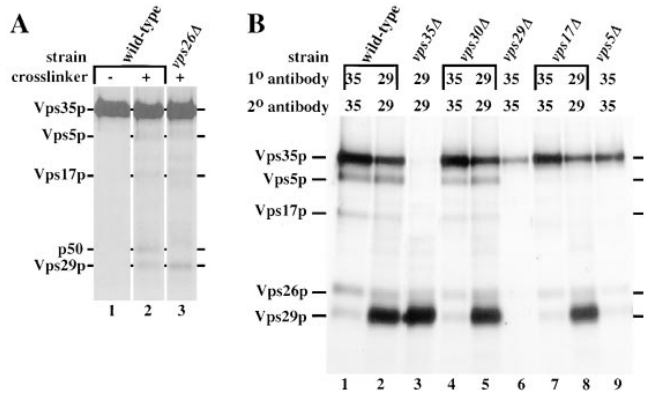

Figure 3. Identification of p50 and assembly of the multimeric complex in mutant strains. (*A*) Lysates from either wild-type or *vps26*Δ cells that had been pulse-labeled with [^{35}S]methionine were either treated with cross-linker (lanes *2* and *3*) or left untreated (lane *1*). After cross-linking, proteins were precipitated, washed with acetone, and resuspended into immunoprecipitation buffer. Vps35p was immunoprecipitated, and the proteins were resolved on an 8% SDS polyacrylamide gel. The band referred to as p50 (Vps26p) is missing in lysates prepared from *vps26*Δ cells, and yet Vps35p is still able to associate with Vps29p, Vps5p, and Vps17p. (*B*) Wild-type cells (SEY 6210) or *vps35* (EMY18), *vps30* (JCY3000), *vps29* (PSY1-29), *vps17* (KKY11), or *vps5* (BHY152) mutants were converted to spheroplasts, labeled with [^{35}S]methionine, chased, and then lysed with the Hepes lysis buffer. The lysate was cleared by centrifugation at 2,000 rpm in a microfuge, and then cross-linker was added to a final concentration of 2 mM. Cross-linking proceeded at room temperature for 30 min, and was then stopped by adding 100 μl of TCA and transferring the samples to ice. The proteins were precipitated and washed with acetone, and a lysate in immunoprecipitation buffer was prepared as before. Vps35p or Vps29p was immunoprecipitated from the lysates using double immunoprecipitations under nonreducing conditions. Interaction of Vps35p with Vps5p/Vps17p required Vps29p, and likewise Vps29p could not interact with Vps5p/Vps17p in a *vps35* mutant.

cleared of unbroken cells was used for these cross-linking experiments. When lysates from wild-type cells treated with cross-linker were immunoprecipitated with antibodies against Vps35p and Vps29p (Fig. 3 *B*, lanes *1* and *2,* respectively), the same pattern of bands was observed as seen in Fig. 2 *A*. When a lysate of the *vps35*Δ strain was cross-linked and Vps29p was immunoprecipitated, Vps29p was not cross-linked to Vps5p/Vps17p (Fig. 3 *B,* lane *3*). Similarly, when the lysate from a *vps29*Δ strain was cross-linked and Vps35p was immunoprecipitated, Vps35p no longer cross-linked to Vps5p/Vps17p or Vps26p (Fig. 3 *B*, lane *6*). In contrast, however, deletion of *VPS30* (Fig. 3 *B,* lanes *4* and *5*) had no effect upon the ability of the complex to assemble. Deletion of either *VPS17* (Fig. 3 *B,* lanes *7* and *8*) or *VPS5* (Fig. 3 *B,* lane *9*) results in the loss of the interaction between Vps35p and Vps5p/Vps17p (Fig. 3 *B,* lanes *7* and *9*). Likewise, Vps29p no longer interacts with Vps5p/Vps17p (Fig. 3 *B,* lane *8*). Interestingly, it appears that the interaction between Vps35p and Vps29p is unaffected in either the *vps17* or *vps5* mutants (Fig. 3 *B,* lanes *7–9*), and Vps35p/Vps29p can still interact with Vps26p. These results hint at the possibility that two distinct subcomplexes assemble together to form a multimeric complex. These two subcomplexes appear to comprise a Vps35p/Vps29p/Vps26p complex and a Vps5p/Vps17p complex.

Vps26p is Required for 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 also 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 *vps26*Δ 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 *vps26*Δ cells ~90% of Vps10p is found in the vacuole-enriched P13. This means that *vps26*Δ 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 *vps26*Δ 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).

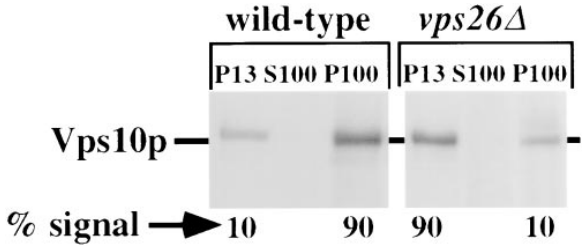

Figure 4. Vps26p is required for the correct localization of Vps10p. Wild-type (SEY 6210) or *vps26* (MSY2600) mutant cells were converted to spheroplasts and labeled with [^{35}S]methionine for 15 min. Excess cold methionine/cysteine was then added, and the cells were chased for 45 min, after which 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 [^{35}S]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 with 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.

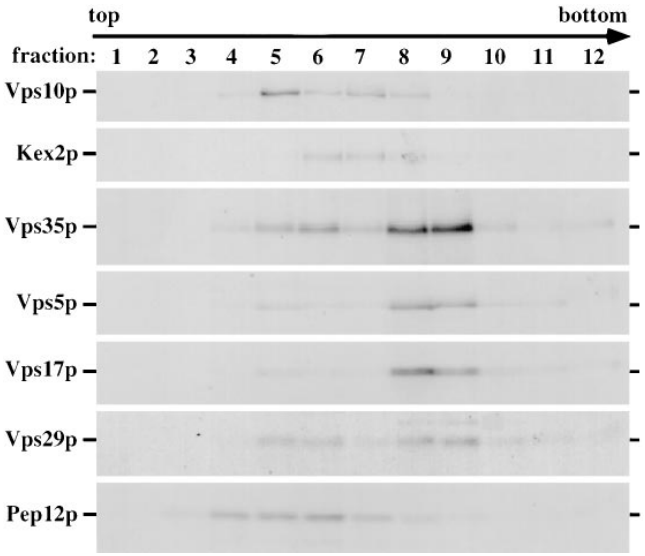

Figure 5. Colocalization of Vps35p and Vps5p in a dense membrane fraction. P100 membranes from [^{35}S]methionine-labeled wild-type cells (SEY 6210) were isolated by differential centrifugation. The membranes were then loaded onto a 10–60% sucrose gradient and spun to equilibrium. Fractions collected from the gradient were precipitated with TCA and washed with acetone, and various proteins were recovered by immunoprecipitation. Vps35p and Vps29p are associated with two discrete pools of membranes. The lighter pool contains the endosomal t-SNARE Pep12p, while the denser population of membranes appears denser than the late-Golgi marker Kex2p. Vps35p (and Vps29p) colocalizes with Vps5p and Vps17p in the denser fractions.

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. 6 *A*), 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

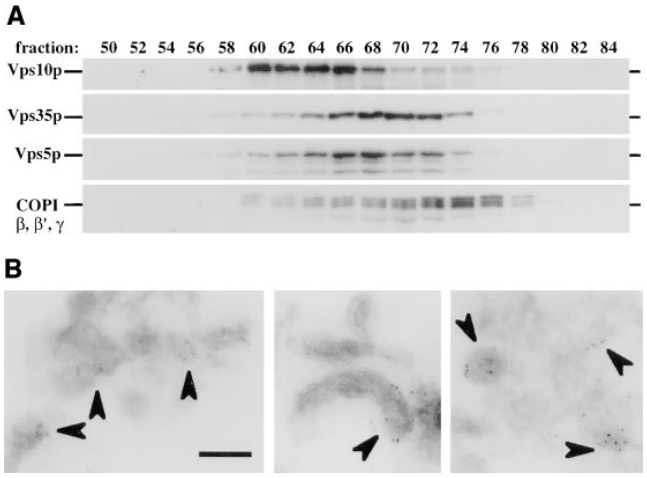

Figure 6. Analysis of the size of the membranes on which Vps35p and Vps5p associate. (*A*) 100,000 *g* membranes were prepared from wild-type cells carrying a myc-tagged version of Vps10p on the chromosome (MSY10-21). The membranes were resuspended into Hepes lysis buffer and loaded onto a sephacryl S1000 column preequilibrated with Hepes lysis buffer. The membranes were eluted using the Hepes lysis buffer, and ~2.5-ml fractions were collected. Fractions were analyzed for the presence of Vps35p and Vps5p (and other proteins) by Western blotting. The membranes with which Vps35p and Vps5p are associated elute after membranes that contain Vps10p (and Kex2p), but before COPI/coatomer-coated vesicles. (*B*) A collage of images of anti-HA labeled membranes. A P100 membrane fraction from wild-type cells (SEY 6210) expressing pAH31 (HA-tagged *VPS5*) (Nothwehr and Hindes, 1997) was fixed with 2% paraformaldehyde and then prepared for cryosectioning. Frozen thin sections were labeled with anti-HA monoclonal antibodies, which were followed by anti-mouse antibodies coupled to 5-nm colloidal-gold. Labeling was predominately restricted to vesicular and tubulovesicular structures. Bar, 100 nm.

COPI/coatomer was determined. COPI-coated vesicles have been shown to have a size of 40–60 nm (Ostermann et al., 1993; Serafini et al., 1991), and eluted from the sizing column in fractions 66–76, although the bulk of the signal is detected in the later fractions 72–76. Thus, it appeared that Vps35p and Vps5p were present on vesicular membranes slightly larger than COPI/coatomer-coated vesicles. The fractionation of Vps35p and Vps5p in fractions smaller than Golgi or endosomes was not the result of fragmentation of membranes during homogenization, as P100 membranes prepared from osmotically lysed cells fractionated in an identical fashion (data not shown).

Fractions that contained the bulk of the Vps35p/Vps5p signal were pooled and processed for morphological analysis by EM, and were found to contain numerous small vesicles (data not shown). Attempts to label these vesicles with antisera against Vps35p, Vps5p, Vps17p, or Vps29p were not successful; however, as an alternative approach, a P100 membrane fraction from cells expressing an epitope-tagged *VPS5* gene was subjected to cryoelectron microscopy. Frozen-thin sections were labeled with anti-HA antibodies and 5 nm anti-mouse colloidal gold. The gold particles were found to decorate vesicular and tubulovesicular structures of 80–120 nm (Fig. 6 *B*).

The distribution of Vps5p in vivo was investigated by using an epitope-tagged *VPS5* gene. This has been shown to complement the *vps5*Δ mutant, and when expressed in a class E *vps* mutant is observed by immunofluorescence to associate with the exaggerated prevacuolar endosome present in class E *vps* mutants (Nothwehr and Hindes, 1997). Therefore, cells expressing the tagged Vps5p were fixed and prepared for cryoelectron microscopy. Frozen-thin sections were labeled with a monoclonal anti-HA antibody, which was followed by 5-nm colloidal gold coupled to anti-mouse antisera. In Fig. 7, an electron micrograph of a *vps4* cell expressing the HA-tagged Vps5p is shown. The prevacuolar endosome is decorated with 5-nm gold particles denoting the presence of Vps5p (Fig. 7 *A*). Strikingly, the labeling is localized to specific regions in the prevacuolar endosomal membrane, and appears most concentrated at the swollen rims of the cisternae that comprise the prevacuolar endosome in a class E mutant (Fig. 7 *B, inset*) (Babst et al., 1997; Rieder et al., 1996). This localized distribution of Vps5p is in contrast to the localization of the vacuolar ATPase, which is found evenly distributed throughout the membranes in a class E mutant (Rieder et al., 1996).

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. Fractions 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-

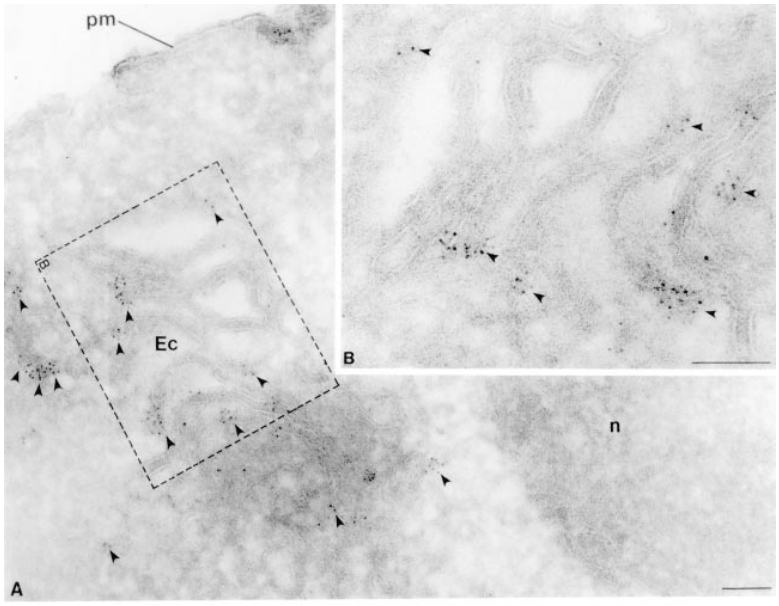

Figure 7. Immunolocaliztion of Vps5p by EM. *vps4* cells (SEY 4-1) harboring the plasmid pAH31 (HA-tagged *VPS5*) (Nothwehr and Hindes, 1997) were fixed and prepared for cryoelectron microscopy. (*A*) Frozen thin sections were labeled with the anti-HA antibody to localize Vps5p, and were followed by 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.

periments (Figs. 2 and 3 *B*). It appears that Vps35p and Vps29p also form a complex (~230 kD) larger than the sum of their two respective molecular masses (110 kD + 30 kD). It is possible that Vps26p is also part of the Vps35p/Vps29p complex, although the lack of antiserum against Vps26p precludes direct testing of this hypothesis. Adding 40 kD of Vps26p to the Vps35p/Vps29p complex would bring the size of the complex closer to 230 kD. Furthermore, in double mutants of *vps29*Δ/*vps26*Δ there was no detectable Vps35p by Western blotting, and the protein appeared to be very unstable, with a half-life of ~30 min (data not shown). Together with data from the cross-linking experiments, these findings support the notion that two subcomplexes of Vps35p/Vps29p/Vps26p and Vps5p/Vps17p, respectively, assemble together on an endosomal membrane.

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 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 Hepes 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 *vps17*Δ 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 *vps29*Δ 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 *vps17*Δ cells was fractionated. Even though Vps5p stably associates with Vps17p (Horazdovsky et al., 1997), cytosolic Vps5p from *vps17*Δ 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 Seaman et al., 1997
Vps29p	0	80	20	This study
Vps26p	0	0–30	70–100	Bachhawat et al., 1994 This study (data not shown)
Vps5p	0	~50	~50	Horazdovsky et al., 1997 Nothwehr and Hindes, 1997
Vps17p	0	~25	~75	Kohrer and Emr, 1993

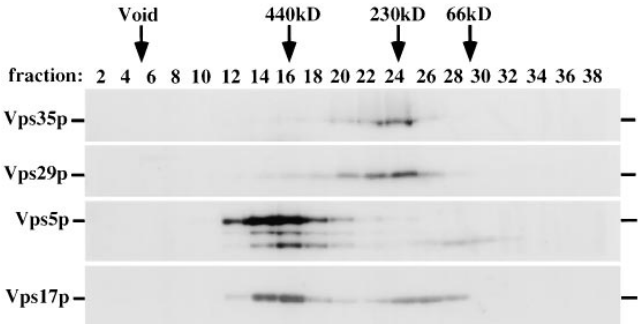

Figure 8. Sizing the components of the multimeric complex reveals the existence of two distinct subcomplexes. P100 membranes prepared from wild-type cells (SEY 6210) were stripped by resuspending the pellet in Hepes lysis buffer containing 250 mM NaCl. The membranes were pelleted by centrifugation at 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.

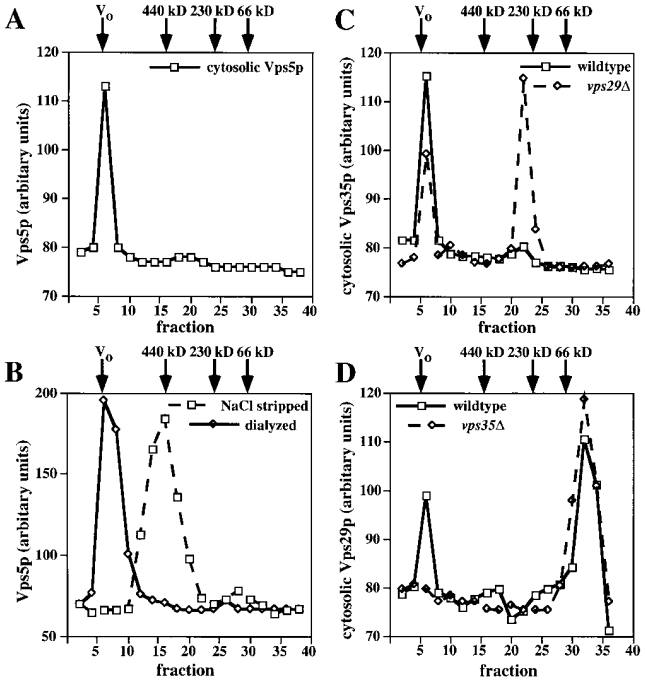

Figure 9. Cytosolic Vps5p assembles into a large (>10^6 D) complex. (*A*) Cytosol (100,000 *g* supernatant) prepared from wild-type cells (SEY 6210) lysed in Hepes lysis buffer was fractionated on a sephacryl S300 column. Fractions collected were precipitated and analyzed for the presence of Vps5p by Western blotting. Resulting autoradiograms were scanned and quantitated using National Institutes of Health Image (version 1.61) software. Vps5p is found almost entirely in the void volume fraction, suggesting that it is part of a complex of >10^6 D. (*B*) Vps5p that had been stripped from P100 membranes using 250 mM NaCl (as in Fig. 8) was dialyzed against Hepes lysis buffer to remove the salt. The supernatant was briefly spun at 13,000 *g,* and was then fractionated on a sephacryl S300 column. Fractions were collected, precipitated, and analyzed by Western blotting. The autoradiogram was quantitated as above. Vps5p is able to assemble into a large (>10^6-D) complex, and elutes in the void volume fraction (*solid line*). This contrasts with the size of Vps5p when fractionated in the continued presence of 250 mM NaCl (*broken line*; this data is the quantitation of the Vps5p signal in Fig. 8). 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 *vps29*Δ 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 *vps35*Δ (EMY 18) cells, 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.

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 degradtion, 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

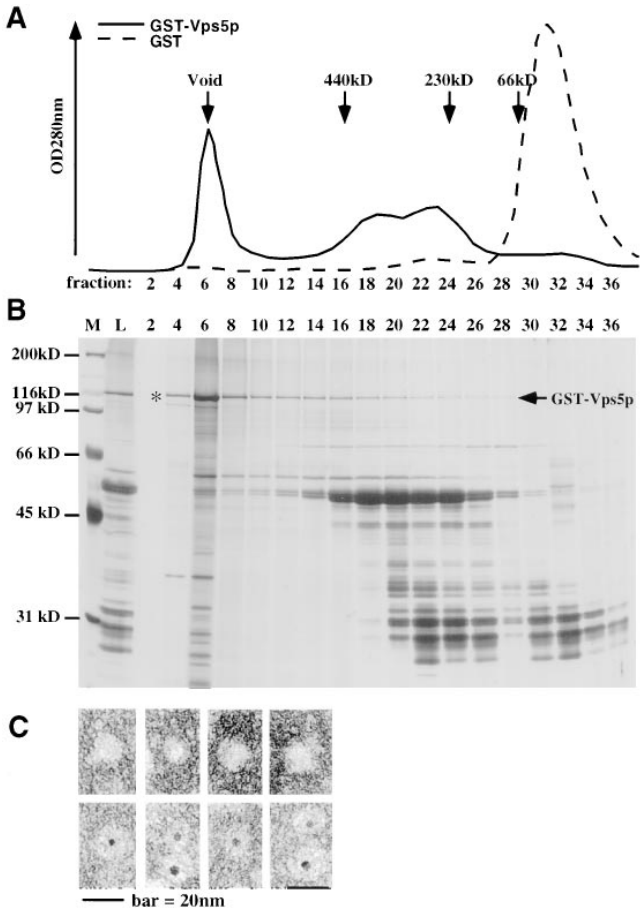

Figure 10. Recombinant Vps5p shows self-assembly activity. (*A*) Affinity-purified recombinant GST-Vps5p and GST alone was dialyzed against the Hepes lysis buffer before being loaded onto a sephacryl S300 gel filtration column. The elution profile of both GST-Vps5p (*solid line*) or GST (*broken line*) is shown. (*B*) The GST-Vps5p–containing fractions from the sephacryl S300 column were analyzed by SDS-PAGE. Lane *M* denotes the molecular mass markers, and lane *L* is the GST-Vps5p before fractionation on the sephacryl S300 column. The void volume fraction (*6*) contains predominately full-length GST-Vps5p, while the abundant breakdown products of the recombinant GST-Vps5p elute later. (*C*) The void volume fraction was adsorbed to a formvar/carbon-coated grid and negatively stained with uranyl acetate for examination by electron microscopy (*upper four micrographs*). In the lower four micrographs, the void volume fraction was labeled with anti-Vps5p antisera and 5 nm anti-rabbit colloidal gold before negative staining. The GST-Vps5p was found to have assembled into a homogeneous population of 15–20-nm spherical particles.

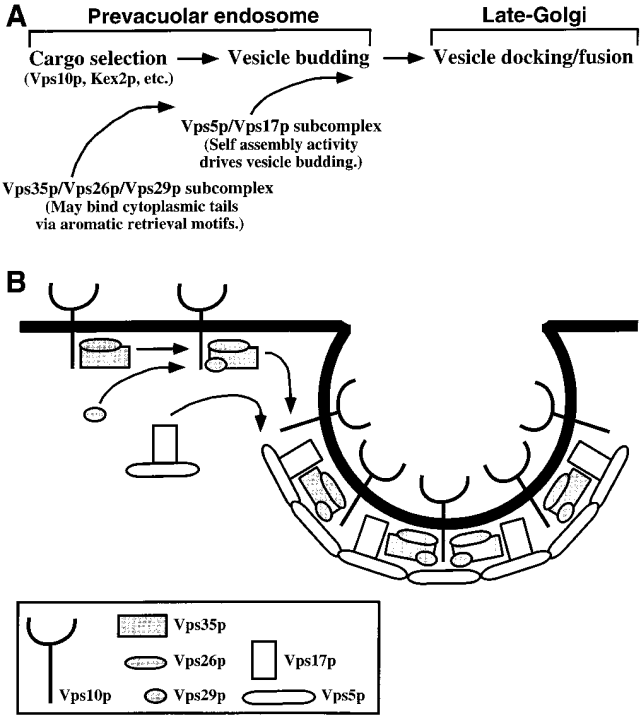

Figure 11. A schematic model for the mechanism of retromer-mediated vesicle formation at the endosome. (*A*) The proposed sequence of events involves Vps35p interacting with cytoplasmic tails of cargo such as Vps10p and Kex2p. Vps29p then activates Vps35p, causing clustering of the Vps35p and cargo. Vps5p/Vps17p then assemble onto the membrane to drive vesicle formation, possibly with Vps17p providing the membrane attachment activity. (*B*) Schematic of the two subcomplexes of the retromer that assemble together onto the endosome membrane. The inner shell of the retromer coat would be the Vps35p/Vps29p/Vps26p subcomplex, while Vps5p/Vps17p form the outer shell.

homogeneous population of 15–20-nm spherical structures. In Fig. 10 *C,* two panels of four electron micrographs showing the void volume fraction bound to the electron microscope grid are shown. In the top four micrographs, the void volume fraction was bound to the grid, and was negatively stained with uranyl acetate, while in the lower four micrographs the bound GST-Vps5p was labeled with anti-Vps5p antisera followed by anti-rabbit coupled to 5-nm colloidal gold before negative staining. Generally one or two gold particles per structure were observed, reflecting a probable lack of access of the antibody to the antigen. The regular homogeneous appearance and the GST-Vps5p particles along with the discrete antibody labeling seen strongly argues for specific assembly of the GST-Vps5p, and not for nonspecific aggregation. Recombinant Vps5p therefore assembles into a high molecular weight complex similar to the endogenous Vps5p.

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 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. To denote its role in retrograde transport from the endosome to the Golgi, we term this complex the retromer.

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

overexpression can bypass the defect observed in cells overexpressing the *vps35dn* 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 Vps5p 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 Vps5p/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 *vps29*Δ/*vps26*Δ 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 *vps5*Δ 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 cofractionate 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 predominately 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 Hβ58, 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 COPI 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, 1993*a*). 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, 1992*b*; Wilsbach and Payne, 1993*b*). 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 (Wilcox et al., 1992; Cereghino et al., 1995; Cooper and Stevens, 1996), and these motifs are similar to a diaromatic motif present in the tail of the M6P receptor that is important for its 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 recogniton 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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