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Vps26p, a Component of Retromer, Directs the Interactions of Vps35p in Endosome-to-Golgi Retrieval

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Endosome-to-Golgi retrieval of the carboxypeptidase Y receptor Vps10p is mediated by a recently discovered membrane coat complex termed retromer. Retromer comprises five conserved proteins: Vps35p, Vps29p, Vps5p, Vps17p, and Vps26p. Vps35p recognizes cargo molecules such as Vps10p and interacts strongly with Vps29p. Vps5p forms a subcomplex with Vps17p and has been proposed to play a structural role by self-assembling into large multimeric structures. The function of Vps26p is currently unknown. We have investigated the role that Vps26p plays in retromer-mediated endosome-to-Golgi transport by analyzing dominant negative alleles of Vps26p. These mutants have identified a crucial region of Vps26p that plays an important role in its function. Functional domains of Vps26p have been investigated by the creation of yeast-mouse hybrid molecules in which domains of Vps26p have been replaced by the similar domain in the protein encoded by the mouse VPS26 gene, Hβ58. These domain swap experiments have shown that Vps26p promotes the interactions between the cargo-selective component Vps35p and the structural components Vps5p/Vps17p.

INTRODUCTION

Protein sorting to the mammalian lysosome is a receptor-mediated vesicular transport step that uses two distinct mannose-6-phosphate receptors (MPRs). These receptors, the cation-independent (CI) MPR and cation-dependent (CD) MPR, rapidly cycle between the *trans*-Golgi network (TGN) and a prelysosomal endosome (Kornfeld and Mellman, 1989; Kornfeld, 1992). Exit of MPRs from the TGN is believed to be mediated by AP-1-containing clathrin-coated vesicles (Le Borgne *et al.*, 1993; Mauxion *et al.*, 1996; reviewed in Robinson, 1997). The molecular mechanisms, in particular the vesicular coat, that drive retrieval of MPRs from the endosome to the TGN are less well understood. Experiments on cells derived from μ1A-deficient mice have indicated a role for AP-1 in retrieval of the CD-MPR from endosomes back to the Golgi (Meyer *et al.*, 2000). Similarly, the transport of endocytosed Shiga toxin back to the TGN has been shown to involve AP-1 (Mallard *et al.*, 1998). Other candidates in mammalian cells for mediating this step are TIP47 (Diaz and Pfeffer, 1998; Krise *et al.*, 1999) and PACS1 (Wan *et al.*, 1998).

In yeast very similar sorting pathways exist. Genetic screens to isolate mutants deficient in sorting of hydrolases to the vacuole have uncovered >50 vacuole protein sorting (*VPS*) genes (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986). The products of these *VPS* genes all function at some point between the late-Golgi and the vacuole (Robinson *et al.*, 1988). A key component in the sorting and transport of vacuolar hydrolases is Vps10p, a type I transmembrane receptor that binds vacuolar hydrolases in the late-Golgi (Marcusson *et al.*, 1994; Cooper and Stevens, 1996). Hydrolases such as carboxypeptidase Y (CPY) and proteinase A are recognized by Vps10p in the late-Golgi. Vps10p is then transported to a prevacuolar endosome where receptor and ligand dissociate (Cereghino *et al.*, 1995; Cooper and Stevens, 1996). Retrieval of Vps10p from the endosome to the late-Golgi is required to maintain sufficient Vps10p in the late-Golgi to sort more CPY and proteinase A (Seaman *et al.*, 1997). Clathrin has been shown to be important in the trafficking of CPY (Seeger and Payne, 1992a) and is also required for the correct localization of Kex2p, a late-Golgi resident endopeptidase factor (Seeger and Payne, 1992b). Clathrin is therefore a candidate for a vesicle coat that mediates endosome-to-Golgi retrieval in yeast; but paradoxically, the deletion of clathrin does not affect transport of CPY to the vacuole, although the effect on Kex2p localization is pronounced (Payne and Schekman, 1989). Neither TIP47 nor PACS1 have homologs in yeast so these proteins may have a specialized function required only in more complicated eukaryotes such as mammalian cells.

Recent studies have uncovered a novel protein complex that is required for retrieval of Vps10p from endosomes to

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Abbreviations used: CPY, carboxypeptidase Y; HA, hemagglutinin; MPR, mannose-6-phosphate receptor; TGN, *trans*-Golgi network.

the late-Golgi in yeast and has been proposed to function as a vesicle coat (Seaman *et al.*, 1998). The complex was dubbed retromer and comprises five Vps proteins: Vps35p, Vps29p, Vps26p, Vps5p, and Vps17p. These proteins are conserved and have homologs in mammalian cells, suggesting that their function is also conserved (Pfeffer, 2001; Renfrew-Haft *et al.*, 2000).

Deletion of any retromer components results in Vps10p becoming mislocalized to the vacuolar membrane. Vps35p is a peripheral membrane protein that colocalizes with Vps10p and will follow Vps10p to the vacuolar membrane in a *vps29* mutant (Seaman *et al.*, 1997). This suggested that Vps35p provides the cargo-selective activity within the retromer complex and indeed studies by Nothwehr *et al*. (1999, 2000) have proved this to be the case. Vps5p and Vps17p form a stable dimer and are both members of the sorting nexin family of proteins (Horazdovsky *et al.*, 1997). In vivo and in vitro Vps5p demonstrates self-assembly activity, and it has therefore been proposed to provide at least some of the mechanical force required to form a vesicle (Seaman *et al.*, 1998). Partially disassembled retromer, which has been stripped from membranes with the use of a salt wash, divides into two subcomplexes. Vps5p/Vps17p comprise one subcomplex, whereas Vps35p/Vps29p comprise the other (Seaman *et al.*, 1998). The presence of Vps26p in either of these subcomplexes is unknown. This division into subcomplexes mirrors the phenotypic differences between the respective mutant strains. *vps35* and *vps29* are class A mutants with morphologically normal vacuoles, whereas *vps5* and *vps17* are class B mutants with severely fragmented vacuoles. *vps26* mutants are class F mutants with an intermediate phenotype exhibiting a partially fragmented vacuole (Raymond *et al.*, 1992).

The precise role of Vps26p is presently unknown. It appears to be a nonstructural member of the complex because a partial retromer complex containing the remaining four subunits can be cross-linked together in *vps26*Δ cells (Seaman *et al.*, 1998). The intermediate phenotype of a *vps26* mutant might suggest a role in bringing together the cargo-selective and structural components of retromer. Here, we present a study of the role that Vps26p plays in endosome-to-Golgi transport in yeast and in retromer function. We have generated dominant negative alleles of *vps26* that can be suppressed by overexpression of *VPS35* and *VPS10*. We have investigated the functional domains of Vps26p by domain swap experiments with the mouse homolog of Vps26p and find that Vps26p is responsible for facilitating the interaction between Vps35p and Vps5p/Vps17p.

MATERIALS AND METHODS

Media, Reagents, and Antibodies

Escherichia coli cells were grown in LB media (supplemented with appropriate antibiotics) prepared by the in-house media kitchen. Bacterial transformations were performed according to Hanahan (1983). Yeast strains were grown on/in either rich media, yeast extract-peptone-dextrose (YPD), or minimal media, yeast nitrogen base-dextrose (YNB), which was supplemented with appropriate amino acids to maintain plasmid selection. Yeast media was also prepared by the in-house media kitchen. Supplementary amino acids and most other general laboratory reagents were purchased from Sigma-Aldrich (Poole, Dorset, United Kingdom). Restriction enzymes were obtained from New England Biolabs (Hitchen, Herts, United Kingdom). Protein A-Sepharose, ^{125}I-protein A, [^{35}S]methionine (Promix), and the sephacryl S300 chromatography media were purchased from Amersham Pharmacia Biotech (St. Albans, Herts, United Kingdom). Anti-CPY antisera was generously provided by Scott Emr, (University of California, San Diego, La Jolla, CA). Antisera against Vps10p, Vps35p, Vps29p, Vps17p, and Vps5p were also provided by Scott Emr. Anti-myc antibodies (9E10) were provided by Paul Luzio (University of Cambridge, Cambridge, United Kingdom). Anti-hemagglutinin (HA) was purchased from Roche (Lewes, East Sussex, United Kingdom).

SDS-PAGE and Western Blotting

SDS-PAGE was performed with the use of the Bio-Rad (Hemel Hempstead, Herts, United Kingdom) Protean II minigel system according to the manufacturer's instructions. For CPY sorting experiments, 8% polyacrylamide gels were used. Fluorography of gels was accomplished with the use of the Amplify reagent from Amersham Pharmacia Biotech. For Western blotting, Hybond-C nitrocellulose membranes were used (Amersham Pharmacia Biotech) in a protocol described in Seaman *et al.* (1998). ^{125}I-protein A was used for detection and was obtained from Amersham Pharmacia Biotech.

Strain Construction, Yeast Manipulation

The yeast strains used in this study are listed in Table 1. Yeast transformations were performed with the use of the alkali cation method as in Elble (1992). The *vps17*Δ strain (MSY1700) was generated with the use of the same *vps17*Δ::*HIS3* construct as was used in Kohrer and Emr (1993). Polymerase chain reaction (PCR) primers that flanked the *VPS17* open reading frame were used to amplify the *vps17*Δ::*HIS3* construct from KKY11 yeast. The resulting PCR product was used to transform SEY6210 cells to create a new *vps17*Δ strain. The MSY2629 and MSY2605 strains were generated by deletion of *VPS26* in the PSY1-29 or BHY152 backgrounds, respectively. Deletion of *VPS26* was achieved in the same way as for MSY2600 (Seaman *et al.*, 1998). MSY5211 was generated by deletion of *VPS5* in the SEY6211 background. This deletion was achieved by the same method used in Horazdovsky *et al.* (1997). The strain MSY5211 was mated with PSY1-29 and diploids were selected on −ade, −lys minimal media. Diploids were then sporulated and the tetrads were broken by digestion with glusulase (Sigma) and repeated extractions with ether. Spores were plated onto rich media. After some colony growth, the cells were replica plated onto −his minimal media. Double knockout mutants were identified by PCR of genomic DNA prepared from HIS-positive candidates.

Isolation of vps26 Dominant Negative Mutants

vps26 dominant negative mutants were generated and screened for in an identical manner to the *vps35* dominant negative mutants described in Seaman *et al.* (1998). Briefly, flanking oligonucleotide primers (designed to anneal ~200 bp on either side of the coding region) were used to PCR through *VPS26* in a PCR reaction in which dATP was limiting generating PCR products with mutations randomly distributed over the entire length of *VPS26*. *VPS26* in the 2 μ vector pRS426 was digested with *Hin*dIII to excise ~70% of the *VPS26* protein coding region. The gapped plasmid was gel purified and transformed along with the PCR product into BHY10 cells. Transformants were selected for a −ura minimal media. Approximately 20,000–30,000 transformants were replica plated onto yeast extract-peptone-fructose (YPF) plates and then screened for secretion of the CPY-Invertase reporter protein with the use of the colorimetric plate assay described in Paravicini *et al.* (1992). Colonies that gave a signal were subjected to another round of screening by plate assay and then were tested for CPY secretion by overlaying the colonies with nitrocellulose and then Western blotting with anti-CPY antibodies. Plasmids were rescued from colonies that gave strong signals by both assays and then retransformed into BHY10

Table 1. Yeast strains used in this study

Strain	Genotype	Source or reference
SEY6210	*MATα leu2-3,112 ura3-52 his3*Δ200 t*rp1*-Δ901 *lys2*-801 *suc2*-Δ9	Robinson *et al.*, 1988
SEY6211	*MAT*a *leu2-3,112 ura3-52 his3*Δ200 t*rp1*-Δ901 *ade2-101 suc2*-Δ9	Robinson *et al.*, 1988
BHY10	SEY6210 *leu2-3,112*::*pBHY11*	Horazdovsky *et al.*, 1994
MSY2600	SEY6210 *vps26*Δ::*LEU2*	Seaman *et al.*, 1998
MSY10-21	SEY6210 *VPS10*::*VPS10-myc*	Seaman *et al.*, 1998
EMY18	SEY6210 *vps35*Δ::*HIS3*	Seaman *et al.*, 1997
PSY1-29	SEY6210 *vps29*Δ::*HIS3*	Seaman *et al.*, 1997
BHY152	SEY6210 *vps5*Δ::*HIS3*	Horazdovsky *et al.*, 1997
EMY3	SEY6210 *vps10*Δ::*HIS3*	Marcusson *et al.*, 1994
SEY4-1	SEY6210 *vps4-1*	Robinson *et al.*, 1988
KKY11	SEY6211 *vps17*Δ::*HIS3*	Kohrer and Emr, 1993
MSY1700	SEY6210 *vps17*Δ::*HIS3*	This study
MSY2629	PSY1-29 *vps26*Δ::*LEU2*	This study
MSY5211	SEY6211 *vps5*Δ::*HIS3*	This study
MSY2905	*vps5*Δ::*HIS3 vps29*Δ::*HIS3*	This study
MSY2605	BHY152 *vps26*Δ::*LEU2*	This study

cells for confirmation of plasmid linkage to phenotype. Eighteen plasmids gave significant signals and demonstrated plasmid linkage to the CPY secretion phenotype. The six plasmids conferring the strongest dominant negative phenotype were sequenced to identify the sites of the mutations.

Plasmid Construction/DNA Manipulation

Standard cloning techniques as described in Sambrook *et al.* (1989) were used for routine DNA manipulation, subcloning, and plasmid construction. Gel isolation of DNA fragments was accomplished with the use of the Qiaex II kit from Qiagen (Crawley, West Sussex, United Kingdom) according to manufacturer's instructions. The cloning of *VPS26* is reported in Seaman *et al.* (1998). *VPS26* in pRS416 was excised from the plasmid by *Kpn*I, *Not*I digestion and then subcloned into pRS426 at the *Kpn*I, *Not*I sites. Subsequently, the dominant negative *vps26* alleles, *vps26-5* and *vps26-10*, were moved into both pRS416 and pRS424 by *Kpn*I, *Not*I digestion. The two mutations present in *vps26-5* were separated by digestion of *vps26-5*-pRS424 with *Bam*HI, yielding a 1.2-kbp fragment, which contained the S173P mutation. This fragment was cloned into wild-type *VPS26*-pRS424, which had been digested with *Bam*HI. This yielded the *vps26-5b* allele. The *Bam*HI fragment liberated from wild-type *VPS26*-pRS424 was cloned into the *Bam*HI digested *vps26-5*-pRS424, which contained the I75T mutation. This yielded *vps26-5a*. In a similar manner, the two mutations present in *vps26-10* were separated by digesting *vps26-10*-pRS424 with *Pst*I to yield a 0.9-kbp fragment, which was subcloned into wild-type *VPS26*-pRS424.

The site-directed mutants (I172A, S173A, and K174A) were generated by PCR and gene sequence overlap extension. Oligonucleotide primers in which the respective site-directed mutation was contained were used along with the *VPS26* flanking primers in PCR reactions in which the N-terminal and C-terminal halves of *VPS26* were amplified. The PCR products from this first round would have contained the desired mutation and were designed to overlap. Therefore, in the second round of PCR, the products from the first round were used as templates, which would anneal to each other in the overlapping area. With the use of the flanking primers, the entire length of *VPS26* was amplified with the site-directed mutations being incorporated. DNA sequencing was used to confirm the presence of the site-directed mutations.

The *vps26*-Hβ58 hybrid molecules were created as follows. The carboxyl-terminal half of *VPS26* was excised by *Pst*I-*Xba*I digestion and subcloned into pBluescript (Stratagene) to generate p26C. Plasmid pE30.1, which contains the full-length cDNA for Hβ58 (Lee *et al.*, 1992), was digested with *Bcl*I and *Sty*I to excise the carboxyl-terminal third. This fragment was subcloned into p26C that had been digested with *Bcl*I and *Xba*I to produce p26C58. The *vps26*ΔC construct was generated by blunting the *Bcl*I and *Xba*I sites and then religation to generate p26ΔC. The HA-tagged *VPS26* (pVPS26-HA) (Seaman *et al.*, 1998) was cut with *Eco*RV to release a 1-kbp fragment that was subcloned into *Eco*RV digested p26C58 to produce the full-length construct p26HA58. The full-length construct was then moved into suitable yeast expression vectors (pRS316 and pRS426) by excision with *Kpn*I and *Sac*I. Removal of the HA tag was achieved by *Bam*HI digestion and subsequent religation of 26HA58 in pRS316. Wild-type *VPS26* was digested with *Eco*RV, and the resulting fragment was cloned into p26ΔC that was then subcloned into pRS316 and pRS426 to generate p26ΔC-316 and p26ΔC-426, respectively.

To express Hβ58 in yeast, the Hβ58 gene was amplified with the use of primers that annealed at the 5′ end close to the start methionine and at the 3′ end ~500 bp downstream of the stop codon. The PCR product was cloned with the use of the PCR blunt vector with the use of the Zero Blunt kit (Invitrogen, Carlsbad, CA). Hβ58 was then excised with *Hin*dIII digestion, blunted, and then digested with *Xho*I. This fragment was cloned into *VPS5*-pRS424, which had been digested with *Nco*I, blunted, and then digested with *Xho*I to remove the *VPS5* coding region but leaving the *VPS5* promoter intact. The Hβ58-26-58 hybrid was created by a three-step gene sequence overlap extension technique. In the first step, the amino and carboxyl-terminal domains of Hβ58 were amplified by PCR with the use of primers that contained 5′ sequences designed to anneal to the central region of *VPS26*. The central region of *VPS26* was amplified with primers with 5′ sequences designed to anneal to the amino- and carboxyl-terminal regions of Hβ58. In the second step, the amino-terminal region of Hβ58 was mixed with the central region of *VPS26* and a PCR reaction was performed to generate an Hβ58-26 fusion. This product was mixed with the Hβ58 carboxyl-terminal domain in the third step and again a PCR reaction was performed, generating the construct Hβ58-26-58. This was subsequently subcloned into VPS5-pRS424 as described above.

A glutathione *S*-transferase (GST)-Vps26p fusion protein was used for generation of an antisera against Vps26p. *VPS26*-pRS424 was digested with *Bam*HI and the 1.2-kbp fragment was gel purified. This was subcloned into pGEX 4T-2 (Amersham Pharmacia Biotech), which had been digested with *Bam*HI. The resulting construct (pGEX-26) was transformed into XL1Blue *E. coli* for expression of the GST-Vps26p fusion protein.

Generation of Anti-Vps26p Antisera

The GST-Vps26p fusion protein (see above) was expressed as an insoluble fusion protein, and therefore inclusion bodies were isolated from the bacteria with the use of the method described in Page and Robinson (1995). Inclusion bodies were subjected to preparative SDS-PAGE to isolate the GST-Vps26p fusion protein. Rabbit immunization was performed with the use of a standard immunization protocol with 1 mg of antigen per immunization. The antisera was affinity purified with the use of GST-Vps26p coupled to cyanogen bromide-activated Sepharose (Amersham Pharmacia Biotech) as an affinity column.

CPY Sorting Assays

Two CPY sorting assays were used in this study. When measuring the effect of the dominant negative *vps26* mutants upon CPY sorting in wild-type cells, an assay that separated intracellular and extracellular fractions after the chase period was used. This assay is described in detail in Seaman *et al.* (1997, 1998). When measuring the ability of the *vps26* mutants to complement the CPY sorting defect in *vps26*Δ cells, a whole cell assay is used. The whole cell assay is described in detail in Seaman *et al.* (1997).

Subcellular Fractionation

To investigate the localization of Vps10p and membrane association of Vps35p, a simple subcellular fractionation procedure was used. This assay has been used extensively in Seaman *et al.* (1997, 1998). Briefly, yeast grown in selective media were converted to spheroplasts by digestion with zymolyase (Seikagaku, Tokyo, Japan). The cells were then pulse labeled for 15 min. Chase (excess methionine and cysteine) was added and the cells were incubated for a further 45 min. After centrifugation, the cells were osmotically lysed in cytosol buffer (20 mM HEPES-KOH, pH. 7.0, 50 mM potassium acetate, 2 mM EDTA, 0.2 M sorbitol). The lysate was cleared by centrifugation for 5 min at 2000 rpm in an Eppendorf microfuge. The cleared lysate was then spun at 13,000 rpm in the microfuge and the pellet (P13) was retained. The supernatant was further centrifuged at 100,000 × *g* with the use of a Beckman benchtop ultracentrifuge and TLA100.3 rotor. The supernatant (S100) and pellet fractions (P100) were retained. All fractions were precipitated with 10% trichloroacetic acid (TCA), washed with acetone, and then solubilized in 100 μl of cracking buffer (50 mM Tris-HCl, pH. 7.4, 6 M urea, 1% wt/vol SDS). After dilution with 1 ml of Tween 20 Tris-buffered saline solution (Tween 20 TBS) (50 mM Tris-HCl, pH. 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.5% vol/vol Tween 20) and centrifugation at 13,000 rpm for 10 min, the lysates were immunoprecipitated with antisera directed against the protein specified.

Stability Assays

Vps10p and Vps35p stability assays are based upon the Vps10p clipping assay described in Cereghino *et al.* (1995). Cells grown in selective minimal media were harvested by centrifugation and then resuspended at 2–3 $OD_{600\,nm}$/ml into fresh media. Cells were pulse labeled for 15 min with [^{35}S]methionine before addition of the 10× chase solution (50 mM methionine, 10 mM cysteine, 5% yeast extract, 10% glucose). The cells were chased over the specified time with 1-ml aliquots of cells being removed at regular intervals. The 1-ml aliquots were precipitated with TCA, washed twice with acetone, and then desiccated. After being resuspended into cracking buffer, the cell pellets were lysed by vortexing with glass beads and then heated to 70°C for 5 min. One milliliter of Tween 20 TBS solution was added and the mixture was spun at 13,000 rpm for 10 min to remove any insoluble material. The supernatant was removed and appropriate antibodies were added.

Native Immunoprecipitations

Cells were grown, spheroplasted, and labeled as described in "Subcellular Fractionations." After collecting the labeled/chased cells by centrifugation, the cells were lysed in cytosol buffer containing 0.5% vol/vol Triton X-100 and protease inhibitors (Sigma). The lysate was cleared by centrifugation at 13,000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube and appropriate antibodies were added. Native immunoprecipitations were carried out at 4°C on a rotating wheel for 90 min. Protein A-Sepharose was then added (70 μl of a 20% slurry) and the tubes were returned to the rotating wheel for a further 60 min at 4°C. The protein A-Sepharose was washed four times with 1-ml aliquots of cytosol buffer before being dried in an Eppendorf speed vac. The protein A-Sepharose was then resuspended into 100 μl of cracking buffer and heated to 70°C for 5 min. One milliliter of Tween 20 TBS buffer was added and the mixture was centrifuged to remove insoluble material. The supernatant was transferred to a fresh tube and appropriate antibodies were added. The secondary immunoprecipitation was carried out at 4°C overnight.

Gel Filtration Chromatography

This method is based upon the protocol described in Seaman *et al.* (1998). Wild-type cells were grown in 1 liter of rich media to a density of ~3 $OD_{600\,nm}$/ml. The cells were harvested by centrifugation, washed with 1 liter of water, and then resuspended into 250 ml of spheroplasting buffer (10 mM Tris-HCl, pH. 7.4, 10 mM $CaCl_2$, 1 M sorbitol, 2 mM dithiothreitol, and 1 μg of zymolyase per OD). When ~90% of the cells had been spheroplasted, the cells were spun out by centrifugation at 2000 rpm in a Beckman JLA16.250 rotor. The cells were washed with 250 ml of wash buffer (20 mM HEPES-KOH, pH 7.0, 50 mM potassium acetate, 2 mM EDTA, and 1 M sorbitol). Cells were reisolated by centrifugation and then lysed in 50 ml of cytosol buffer containing protease inhibitors. The lysate was cleared by centrifugation for 10 min at 13,000 × *g* with the use of a Beckman JA20 rotor. The lysate was then further spun at 100,000 × *g* in a Beckman ti70 rotor to generate a P100 membrane fraction. The supernatant was removed and the pellet (P100) was resuspended into 3 ml of cytosol buffer containing 250 mM NaCl. Resuspension was aided by several strokes with a Dounce homogenizer. The membranes were pelleted again and 2 ml of the supernatant was then loaded onto a sephacryl S300 column that was equilibrated in cytosol buffer plus 250 mM NaCl. Proteins were eluted at a flow rate of 0.4 ml/min. Forty fractions were collected (each 1.25 ml in volume). Fifty microliters of the even-numbered fractions were added to 50 μl of 2× SDS-PAGE sample buffer and then boiled for 3 min. The proteins were subjected to SDS-PAGE. Western blotting was used to detect the retromer components.

RESULTS

Isolation of Dominant Negative vps26 Alleles

To investigate the role that Vps26p plays within the retromer complex and in endosome-to-Golgi transport in general, a screen was undertaken to isolate dominant negative mutants of Vps26p. Several dominant negative mutants were isolated that resulted in the secretion of a reporter protein, CPY-Invertase, from the cell. With the use of a colorimetric plate assay the activity of the secreted CPY-Invertase can be easily detected, which facilitates screening through large numbers of yeast colonies. Subsequent sequencing of six mutants with the strongest phenotype revealed that each mutant allele had two or three mutations distributed evenly within the region of Vps26p that was mutated by random PCR mutagenesis (Table 2). Interestingly, a single mutation, the substitution of serine at position 173 by proline was

Table 2. Dominant negative *vps26* mutants

Allele	Mutations
*vps*26-5	I75T, **S173P**
*vps*26-10	**S173P**, V234A
*vps*26-12	S102P, **S173P**
*vps*26-14	K162R, **S173P**, V231A
*vps*26-19	V65A, **K174E**, T198A
*vps*26-25	S97G, **S173P**, D230N

present in five of the six alleles sequenced. The sixth allele had a substitution of lysine at 174 by glutamate. The frequency of the mutations detected at residues 173–174 suggested that this region may be critical for Vps26p function. To test whether the S173P substitution was conferring the dominant negative phenotype, the S173P mutation in the *vps26-5* allele was separated from the other mutation present (I75T) and each of the resulting single mutants was tested for a dominant negative phenotype.

In Figure 1A, wild-type cells have been transformed with wild-type *VPS26* in a multicopy vector, the dominant negative allele *vps26-5* or one of two *vps26* alleles derived from *vps26-5* containing the two mutations, S173P or I75T. The cells were assayed for their ability to sort and deliver CPY to the vacuole. Cells containing either the dominant negative

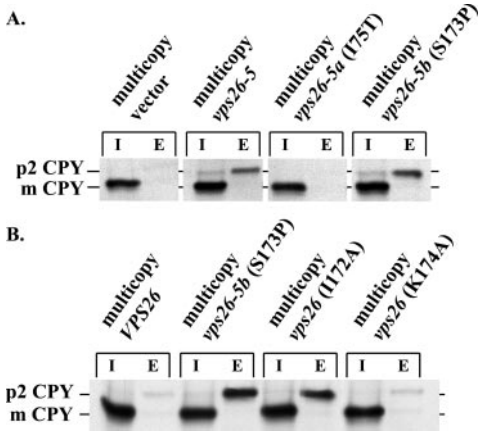

Figure 1. (A) *vps26* S173P mutation confers a dominant negative phenotype. Wild-type (SEY6210) cells were transformed with plasmids to express the dominant negative allele *vps26-5* or two new alleles (*vps26-5a* I75T, *vps26-5b* S173P) derived from *vps26–5* in which the two mutations present were separated. Control cells were transformed with empty vector. The cells were pulse labeled with [^{35}S]methionine for 10 min and then chased for 30 min. After spheroplasting, intracellular and extracellular fractions were separated and CPY was recovered by immunoprecipitation. The dominant negative phenotype is caused by the S173P mutation and not the I75T mutation. (B) Site-directed mutations were engineered into Vps26p producing three new mutants: I172A, S173A, and K174A. These new mutants were tested for dominant negative effects on CPY sorting. Control cells were transformed with wild-type *VPS26* or the *vps26–5b* allele described above. CPY sorting was performed as described above. Only the I172A mutant had a dominant negative phenotype.

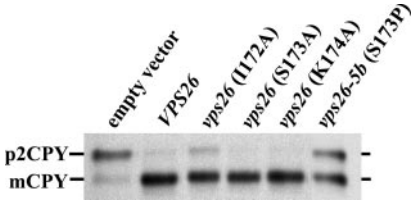

Figure 2. *vps26* S173P and I172A mutants can partially complement the *vps26*Δ mutant. *vps26*Δ cells were transformed with CEN plasmids to express wild-type *VPS26*, or the following mutants; I172A, S173A, K174A, and S173P. CEN plasmids were used to ensure wild-type levels of expression. Cells were pulse labeled for 10 min, chased for 30 min, and then total protein was precipitated with the use of TCA. After washing with acetone, the cells were lysed with the use of glass beads and CPY was recovered from the resulting lysate. The I172A and S173P mutants are partially functional, whereas the S173A and K174A mutants complement the *vps26*Δ as well as the wild-type *VPS26*.

allele *vps26-5* or *vps26* with just a single mutation of S173P both secreted ~45% of the p2 CPY. The wild-type *VPS26* and the I75T mutation had no dominant negative effect. Therefore, the S173P mutation does indeed confer the dominant negative phenotype. An identical result was obtained when the S173P mutation was separated from the V234A mutation present in the *vps26-10* allele (our unpublished data).

To further explore the role that the 172–174 region of Vps26p plays in its function, site-directed mutants were constructed in which the serine, isoleucine, and lysine were each individually changed to alanine. The three new mutants were introduced into wild-type cells on multicopy plasmids and CPY sorting to the vacuole was tested. As shown in Figure 1B, sorting of CPY to the vacuole in cells expressing the I172A mutant is perturbed to a similar extent as it is in cells expressing the S173P mutant. Mutation of the lysine at 174 to alanine did not result in any secretion of p2 CPY from the cell. The S173A mutant also did not secrete any p2 CPY from the cell and was not therefore dominant negative (our unpublished data).

One possibility to explain the lack of phenotype exhibited by the S173A and K174A mutations was that these mutants were in fact null mutants and possessed no residual function. This would result in a lack of dominant negative phenotype and would be indistinguishable from wild-type *VPS26* but would be easily distinguished from wild-type *VPS26* by testing for complementation of the *vps26*Δ mutant. Therefore, the *vps26* alleles described so far were moved into centromeric (CEN) plasmids to be expressed at wild-type levels. *vps26*Δ cells were transformed with the CEN-*vps26* mutants and sorting of CPY to the vacuole was assayed. In Figure 2, the *vps26*Δ cells containing empty vector fail to transport p2 CPY to the vacuole where it would be matured. Hence, the CPY detected is in the p2 form. When the *vps26*Δ cells are complemented by the wild-type *VPS26*, CPY is correctly matured. Cells containing the I172A mutation sorted and matured ~80% of the CPY. The S173A and K174A mutations both sorted CPY as well as the wild-type *VPS26* gene. In contrast, the S173P mutation was only partially functional, resulting in ~50% of the CPY failing to be matured. Clearly, the S173A and K174A mutations are func-

tional and this is why they exhibit no dominant negative effect.

vps26 Dominant Negative Mutants Alter Vps10p Trafficking

Vps26p is part of the retromer complex and is necessary for the retrieval of the CPY receptor Vps10p, from the endosome to the late-Golgi (Seaman *et al.*, 1998). *vps26*Δ mutants have been shown to result in the accumulation of Vps10p in the vacuolar membrane (Seaman *et al.*, 1998). Therefore, the effect that the *vps26* dominant negative mutants have upon Vps10p localization was investigated. Wild-type cells transformed with multicopy plasmids to express either wild-type *VPS26* or the S173P *vps26* dominant negative mutant were converted to spheroplasts, labeled with [^{35}S]methionine, chased and then subjected to a differential centrifugation assay to separate vacuolar membranes from Golgi, endosomal, and vesicular membranes. In Figure 3A, the localization of Vps10p is depicted. Cells expressing the wild-type *VPS26* localize the majority (84%) of the Vps10p to the P100 fraction, which contains Golgi, endosomal, and vesicular membranes. Cells expressing the dominant negative *vps26* resulted in a shift of Vps10p to the vacuole membrane, resulting in 36% of the total Vps10p becoming localized to the vacuolar membrane fraction. This represents an intermediate phenotype with respect to the wild-type localization of Vps10p and its localization in *vps26*Δ cells and is consistent with the intermediate effect upon CPY sorting of the *vps26* dominant negative mutants. Immunofluorescence localization of an epitope-tagged Vps10p in cells expressing either wild-type *VPS26* or the dominant negative *vps26* mutant confirmed that there was no gross change in the localization of Vps10p and no abnormal morphological defects were observed either (our unpublished data).

The effect that the *vps26* mutants were having upon the localization of Vps10p could also be assayed by examining the stability of Vps10p. Class E *vps* mutants such as *vps4* and *vps27*, which both accumulate an exaggerated endosomal compartment, cause Vps10p to be proteolytically clipped (Cereghino *et al.*, 1995; Piper *et al.*, 1996). The effect upon Vps10p stability in cells expressing the dominant negative *vps26* mutants was investigated. Cells were pulse labeled for 15 min and then chased over a period of 90 min with aliquots removed every 30 min. Vps10p was recovered by immunoprecipitation. In Figure 3B the effect of the *vps26* dominant negative mutation upon Vps10p stability is shown. The *vps26* mutant results in Vps10p being clipped with a kinetic half-time of >90 min. In contrast, *vps4* cells clip Vps10p with a half-time of just 30 min. When the *vps26* dominant negative mutant is introduced into *vps4* cells, the kinetics of the clipping of Vps10p appear to be unaffected, indicating that the *vps26* mutant cannot make the defect in Vps10p trafficking present in *vps4* cells worse than it already is. When the S173P dominant negative allele (*vps26–5b*) was introduced into a *pep4* null strain, which lacks the protease proteinase A, no clipping of Vps10p was observed (our unpublished data). This indicates that the clipping of Vps10p does occur in the vacuole and is likely to be the result of a defect in retrieval.

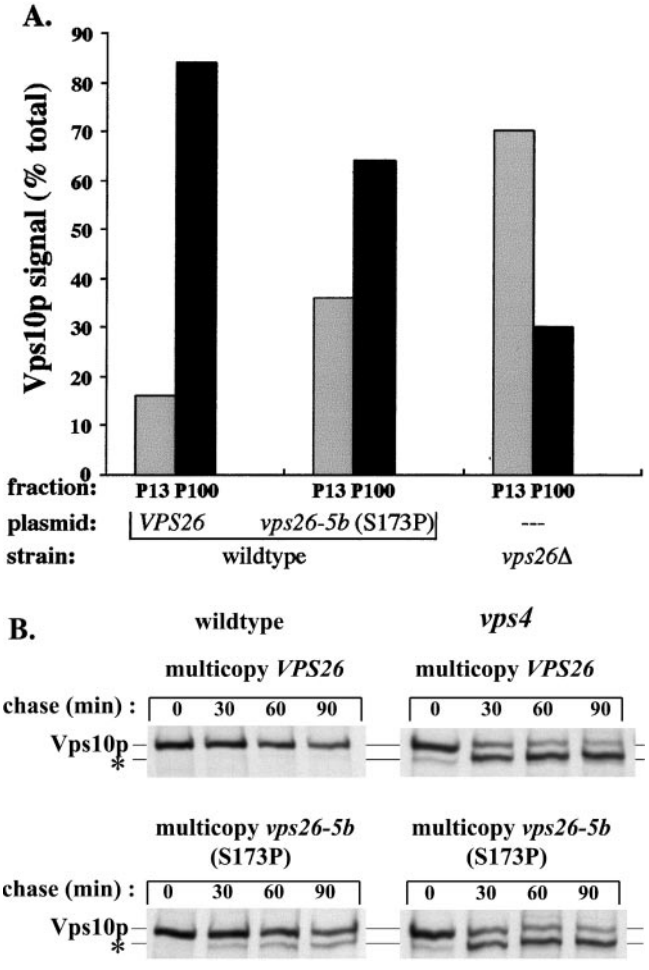

Figure 3. (A) Vps10p is partially mislocalized in cells expressing the *vps26* S173P mutant. Wild-type (SEY6210) cells were transformed with multicopy vectors to express either wild-type *VPS26* or the *vps26* S173P mutant. Control *vps26*Δ cells were left untransformed. Approximately 10 $OD_{600\ nm}$ equivalents of cells were converted to spheroplasts, labeled for 15 min with [^{35}S]methionine, and then chased for 45 min. The cells were spun out and then osmotically lysed in cytosol buffer. Membrane fractions were separated by differential centrifugation. Each fraction was then precipitated with 10% TCA and Vps10p was recovered from the resulting lysate. After SDS-PAGE and fluorography, the autoradiogram was quantitated with the use of NIH Image software. Data from two separate experiments were averaged to produce the graph. (B) Vps10p is unstable in cells expressing the *vps26* S173P mutant. Wild-type or *vps4* cells expressing either wild-type *VPS26* or the *vps26* S173P mutant were labeled with [^{35}S]methionine for 15 min and then chased for up to 90 min with aliquots of cells being removed at 30-min intervals. Lysates were generated with the use of the glass-bead method, and Vps10p was recovered from the resulting lysate and subjected to SDS-PAGE. *vps4* cells and wild-type cells expressing the *vps26* S173P mutant both cause Vps10p to be clipped to the lower molecular weight form (*).

C Terminus of Vps26p Is Critical for Function

The studies on the *vps26* dominant negative mutants revealed that the central region of Vps26p, which contains the

residues 172–174 (highlighted in a box in Figure 4A), was clearly very important for function. Alignment of Vps26p with homologs present in mouse, chicken, slime mold, nematode, and fission yeast (Figure 4A) shows that this central region of Vps26p is well conserved, although the degree of conservation is not as impressive as it is in the carboxyl-terminal third of the protein. We have investigated the functional importance of the conserved and unique regions of Vps26p by constructing a series of yeast-mouse hybrid proteins. Most of these hybrids were unable to complement the *vps26*Δ mutant and several were extremely unstable, indicating a problem with the folding of the hybrid protein. For instance, a truncation of Vps26p in which the carboxy terminus is removed was degraded with a half-life of <10 min (our unpublished data). One construct, however, was very intriguing. Replacing the carboxy terminus of Vps26p with the homologous region of the mouse homolog Hβ58 resulted in a hybrid molecule that was fully functional. This hybrid construct was also generated by Jones and colleagues and was reported to be fully functional in its ability to complement CPY sorting in a *vps26* mutant (Bachhawat *et al.*, 1994). To facilitate detection of the hybrid construct a HA epitope-tagged construct, in which a triple HA tag was inserted between residues 76/77, was used as the basis for this hybrid construct. In Figure 4A the site of the insertion of the HA tag is denoted by an empty triangle, whereas the domain of Vps26p that was swapped with the corresponding domain of the mouse homolog is indicated by the closed triangles. Surprisingly, when we tested the Vps26p-HA-Hβ58 hybrid for its ability to complement CPY sorting in the *vps26*Δ cells we found that it had no complementing activity at all (Figure 4B). Excision of the HA tag, however, restored the function of the Vps26p-Hβ58 hybrid construct. Presence of the tag alone had no effect upon the ability of the Vps26p-HA to complement the *vps26*Δ cells. The HA tag and Hβ58 carboxyl-terminal third therefore appeared to have a synthetic effect upon the function of the Vps26p-HA-Hβ58 fusion. This lack of complementing activity by the Vps26p-HA-Hβ58 fusion was not due to an instability of the hybrid molecule because the protein was made and could be detected at similar levels to wild-type Vps26p (our unpublished data).

Vps26p Interacts with Vps35p

Previous studies have indicated that Vps26p cannot be a key structural component of the retromer complex because the remaining four members can still be cross-linked together in extracts from the *vps26*Δ cells (Seaman *et al.*, 1998). A key function of Vps26p may therefore be to interact with other components of the retromer complex and by doing so direct these other components to perform their discrete function. The *vps26* mutants so far described here have a functionality that ranges from 80% for the I172A mutant through 50% for the S173P mutant to 0% for the Vps26p-HA-Hβ58 hybrid protein. One possibility for these differences may be the ability of the respective mutant proteins to interact with other retromer complex members. To test this, a series of native immunoprecipitations were performed. Cells were converted to spheroplasts, labeled with [^{35}S]methionine, and then chased. Extracts were prepared by lysing the cells in buffer containing 0.5% Triton. Vps26p was immunoprecipitated under native conditions. After several washes, the immunoprecipitated proteins were boiled in cracking buffer and the resulting lysates were reimmunoprecipitated with antibodies against the other retromer complex components. In Figure 5A, Vps26p will coimmunoprecipitate the other four retromer complex members from wild-type extracts. Control extracts from the *vps26*Δ cells similarly treated did not coimmunoprecipitate any of the other retromer complex members (our unpublished data). Interactions between Vps26p and the other retromer complex components required Vps35p as none of the other retromer components coimmunoprecipitated with Vps26p from *vps35*Δ extracts. Extracts from *vps29* cells showed that Vps26p could interact with Vps35p, albeit at a slightly reduced level. Vps29p was, however, clearly required for further interactions with Vps5p/Vps17p. Deletion of either Vps5p or Vps17p resulted in Vps26p coimmunoprecipitating with Vps35p and Vps29p only. A trace amount of Vps5p was detected interacting with Vps26p in extracts from the *vps17*Δ cells.

To test for the effect that deletion of *VPS26* has upon interaction between the other members of the retromer complex, we performed a similar immunoprecipitation assay but used antisera against Vps5p to immunoprecipitate the complex. In Figure 5B, Vps5p will coimmunoprecipitate the other members of retromer (including Vps29p; our unpublished data) from wild-type cells. The control extract from *vps5*Δ cells indicates that the presence of the other retromer components is specific. Deletion of *VPS26* results in a significantly reduced interaction between Vps35p/Vps29p and Vps5p, whereas the interaction between Vps5p and Vps17p is unaffected. Deletion of *VPS29* completely ablates the interaction between Vps35p and Vps5p. This finding is consistent with the original observation that Vps26p is not an essential structural component of retromer and that Vps29p is required for the interaction between Vps35p and Vps5p/Vps17p (Seaman *et al.*, 1998).

With the use of the native immunoprecipitation assay to test for interactions between Vps26p and other members of the retromer complex, the ability of *vps26* dominant negative mutants and the Vps26p-HA-Hβ58 hybrid to interact with the other retromer components was examined. In Figure 5C, the dominant negative *vps26* mutants were coimmunoprecipitated with all of the other four retromer complex members. In contrast, the Vps26p-HA-Hβ58 hybrid, although coimmunoprecipitating both Vps35p and Vps29p to a similar degree as the wild-type protein, was unable to coimmunoprecipitate normal amounts of Vps5p and Vps17p. The Vps26p-Hβ58 and the Vps26p-HA both coimmunoprecipitated the other four members of retromer in a similar manner to wild-type Vps26p. This apparent inability of the Vps26p-HA-Hβ58 to coimmunoprecipitate the Vps5p/Vps17p components of the retromer complex provides a molecular mechanism behind the lack of the *vps26*Δ-complementing activity of the hybrid molecule.

The native immunoprecipitation experiments in Figure 5 indicate that Vps26p interacts most strongly with Vps35p and requires Vps35p for interaction with other members of the retromer complex. This suggests that perhaps Vps26p may modulate Vps35p function in some way, perhaps contributing to the cargo selection role that has been demonstrated for Vps35p (Nothwehr *et al.*, 1999, 2000). Interactions between Vps26p and Vps35p were further investigated by examining the effect of the mutations in Vps26p upon the

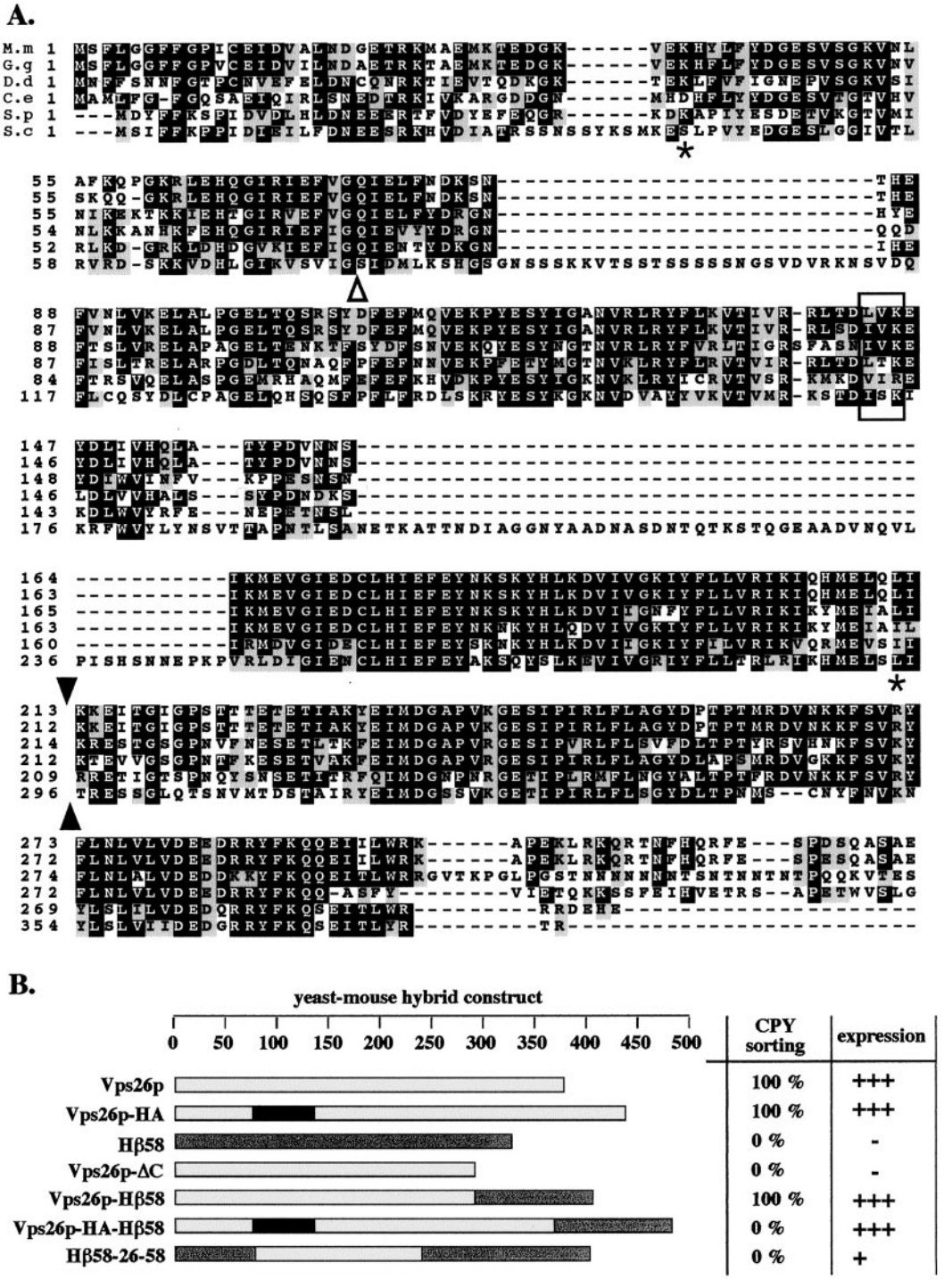

Figure 4. (A) Alignment of Vps26p with various homologs. The protein sequence of Vps26p (bottom line, S.c.) has been aligned with homologs from mouse (M.m), chicken (G.g) dictyolstelium (D.d), worm (C.e) and fission yeast (S.p) to highlight the highly conserved regions such as the C terminus downstream of Vps26p residue 247. The region of Vps26p that was subjected to mutagenesis in the screen for dominant negative mutants is contained between the two asterisks (*). Residues 172–174, which when mutated can give a dominant negative phenotype, are highlighted in a box. The site of introduction of the HA tag is denoted by the open triangle. The domains of Vps26p and the mouse homolog Hβ58 that were swapped are marked by closed triangles. (B) Schematic of the yeast-mouse hybrid constructs and their respective abilities to complement the CPY sorting defect in a *vps26*Δ mutant.

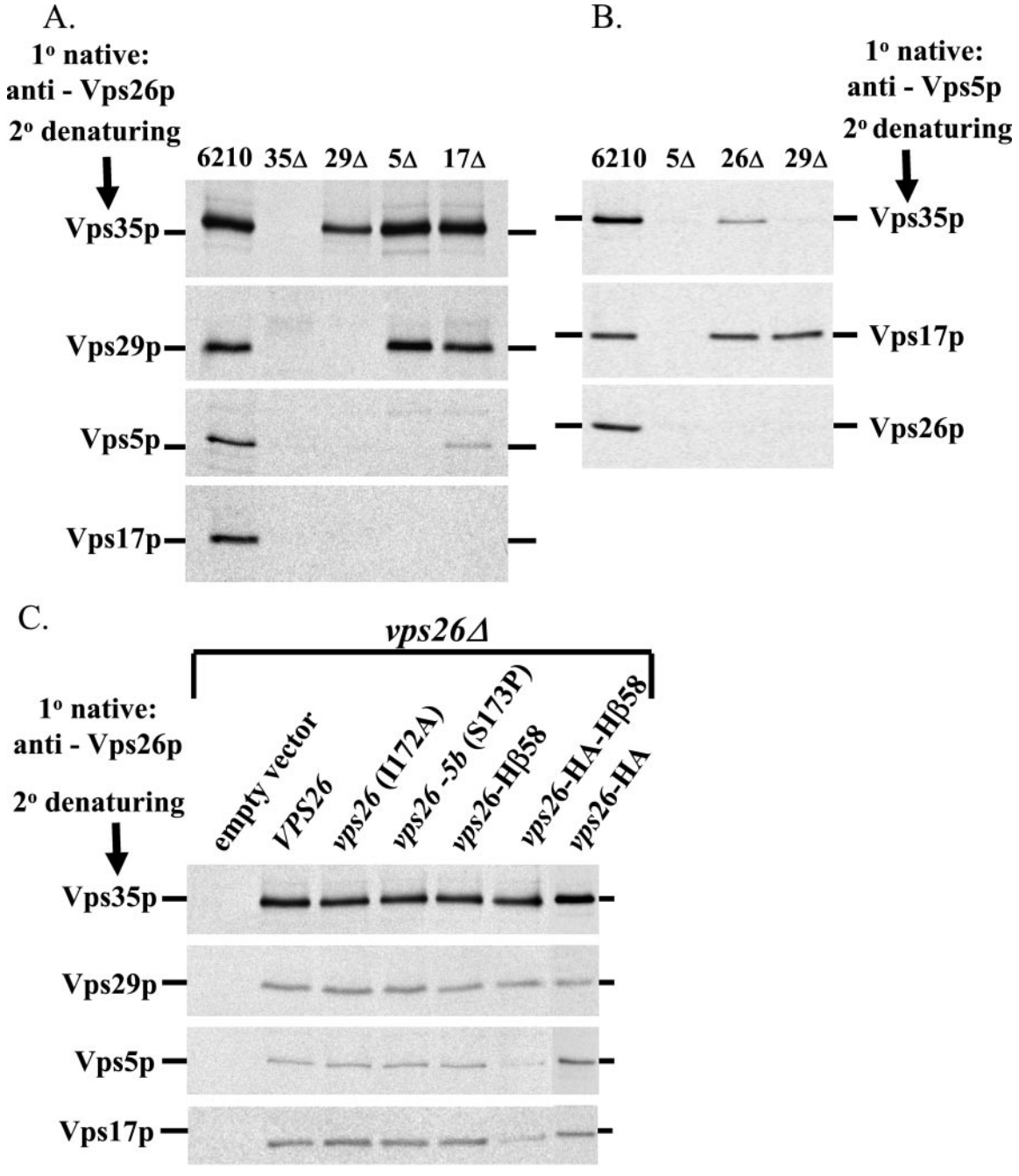

Figure 5. (A) Vps26p can interact directly with Vps35p. Wild-type and retromer deletion mutant cells were converted to spheroplasts, labeled with [^{35}S]methionine for 15 min and then chased for 45 min. After centrifugation, the cells were lysed in cytosol buffer containing 0.5% Triton. The lysate was cleared by centrifugation at 13,000 × *g* and then Vps26p was immunoprecipitated under native conditions. After washes, the primary immunoprecipitates were boiled in urea cracking buffer, and the resulting lysate was reimmunoprecipitated under denaturing conditions with the use of antibodies against the other retromer components. Secondary immunoprecipitates were subjected to SDS-PAGE and fluorography. Vps35p is required for Vps26p to interact with other retromer components. Vps26p will continue to interact with Vps35p and Vps29p in the absence of either Vps5p or Vps17p. (B) Similar to A, Vps5p was immunoprecipitated under native conditions and then other retromer components were reimmunoprecipitated under denaturing conditions. Vps26p is not strictly essential for an interaction between Vps35p/Vps29p and Vps5p/Vps17p, whereas Vps29p is absolutely required. (C) With the use of the technique described in A, the interactions between retromer components and the various *vps26* mutant alleles, including the *VPS26*-Hβ58 hybrids, were examined. The *vps26* S173P and I172A mutants were fully functional with respect to interactions with other retromer components. The Vps26p-HA-Hβ58 hybrid failed to properly coimmunoprecipitate Vps5p/Vps17p. Taken with the data from B, this suggests that Vps26p facilitates interactions between Vps35p/Vps29p and Vps5p/Vps17p.

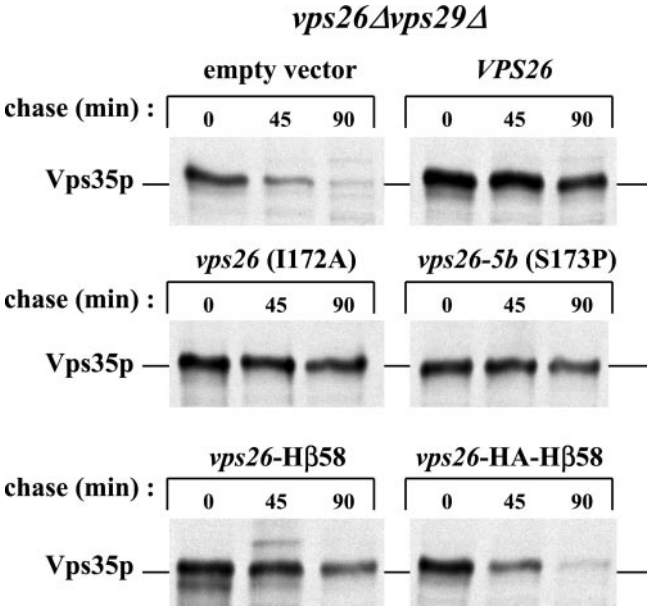

Figure 6. Vps26p-HA-Hβ58 hybrid fails to properly interact with Vps35p. *vps26*Δ*vps29*Δ double mutant cells were transformed with CEN vectors to express wild-type *VPS26*, the I172A and S173P mutants, the Vps26p-Hβ58 hybrid, and the Vps26p-HA-Hβ58 hybrid. Cells were labeled for 15 min and then chased for up to 90 min with aliquots of cells being removed at 0, 45, and 90 min. After TCA precipitation, acetone washes, and glass-bead lysis, the resulting extracts were immunoprecipitated with antibodies against Vps35p. Immunoprecipitates were washed and then subjected to SDS-PAGE and fluorography. The Vps26p-HA-Hβ58 hybrid cannot rescue the stability of Vps35p in the *vps26*Δ*vps29*Δ double mutant, indicating that it cannot properly interact with Vps35p.

stability of Vps35p in cells lacking both Vps26p and Vps29p. It has been previously reported that deletion of *VPS26* and *VPS29* together render Vps35p very unstable (Seaman *et al.*, 1998). No other double mutant combination of the retromer components has this effect (our unpublished data). Therefore, the stability of Vps35p can be used as an assay for interactions between Vps35p and Vps26p. *vps26*Δ*vps29*Δ double mutant cells were transformed with plasmids to express the *vps26* mutants described already. With the use of a protocol similar to that for the stability of Vps10p, the stability of Vps35p was investigated. Cells expressing empty vector result in Vps35p instability and subsequent degradation with a half-time of $<$45 min (Figure 6). The dominant negative mutants I172A and S173P both restore the stability of Vps35p to a similar degree as wild-type *VPS26*. The Vps26p-Hβ58 hybrid also rescues Vps35p stability almost as well as wild-type Vps26p but interestingly the Vps26p-HA-Hβ58 fusion does not rescue Vps35p stability. This suggests that in spite of the ability of the Vps26p-HA-Hβ58 hybrid to coimmunoprecipitate Vps35p, this interaction may in fact be too weak to support the stability of Vps35p in a *vps26*Δ*vps29*Δ double mutant.

VPS35 Can Suppress Dominant Negative vps26 Mutants

The physical interactions that Vps26p has so far demonstrated here favor a role in retromer that facilitates the function of Vps35p. If this is the case, it would be reasonable to expect that the *vps26* dominant negative mutants would be genetically suppressed by overexpression of *VPS35*. Cells transformed with multicopy plasmids to express the dominant negative mutants were also transformed with plasmids to overexpress other retromer components and also *VPS10*. CPY sorting assays were performed. In Figure 7A, the I172A mutant results in secretion of ~45% of the CPY in the p2 form. Cooverexpression of *VPS10*, *VPS35*, and *VPS26* restored CPY sorting such that ~80% of the CPY is correctly matured in the vacuole. Overexpression of *VPS29*, *VPS5*, or *VPS17*, however, did not restore CPY sorting. Similar results were obtained with the S173P mutation, which was also suppressed by overexpression of *VPS10* and *VPS35* (Figure 7B). The suppression of the dominant negative *vps26* mutants by *VPS35* was not the result of a bypass suppression because overexpression of *VPS35* in the *vps26*Δ mutant did not result in any rescue of the CPY sorting defect (our unpublished data). Overexpression of *VPS10* in the *vps26*Δ mutant did result in a small amount of CPY being matured (our unpublished data).

The physical and genetic evidence clearly favors a role for Vps26p in directing or facilitating Vps35p function. Vps35p is peripherally associated with membranes that also contain Vps10p. In previous studies a noted effect of the deletion of *VPS29* was to cause Vps35p to "follow" Vps10p to vacuolar membranes (Seaman *et al.*, 1997). It has also been shown that *vps26*Δ mutants mislocalize Vps10p to the vacuolar membrane fraction (Seaman *et al.*, 1998). When the localization of Vps35p in *vps26*Δ cells was investigated it was noticed that a significant proportion of Vps35p became cytosolic (Figure 8). Furthermore, the remaining membrane-associated Vps35p does not follow Vps10p to the vacuolar fraction as occurs in a *vps29*Δ mutant. This effect upon Vps35p was unique to *vps26*Δ mutants and was not observed in *vps5*Δ, *vps17*Δ, or any combination of double retromer mutants (our unpublished data). We also found that deletion of *VPS26* only affected the membrane association of Vps35p and did not affect the membrane association of Vps5p, Vps17p, or Vps29p (our unpublished data).

These data together argue for Vps26p exerting its effect upon Vps35p. Clearly, Vps26p interacts significantly with Vps35p, but does Vps26p comprise part of a retromer subcomplex and if so, which one? Previously, it has been demonstrated that retromer can be stripped from membranes and divided into two subcomplexes by treatment with 250 mM NaCl. The resulting extract when size fractionated by gel filtration chromatography showed that Vps5p remains associated with Vps17p, whereas Vps35p maintains interactions with Vps29p (Seaman *et al.*, 1998). The behavior of Vps26p under these conditions was previously unknown. In Figure 9 we have investigated what happens to Vps26p when salt stripped from P100 membranes. P100 membranes prepared from wild-type cells were treated with 250 mM NaCl to strip off retromer. The membranes were removed by centrifugation and the soluble proteins were size fractionated on a sephacryl S300 column. As seen previously, Vps5p cofractionates with Vps17p and Vps35p cofractionates with Vps29p. Vps26p elutes from the column predominantly in later fractions, consistent with it being in a monomeric form not associated with other retromer components, although small amounts of Vps26p are present in the fractions that

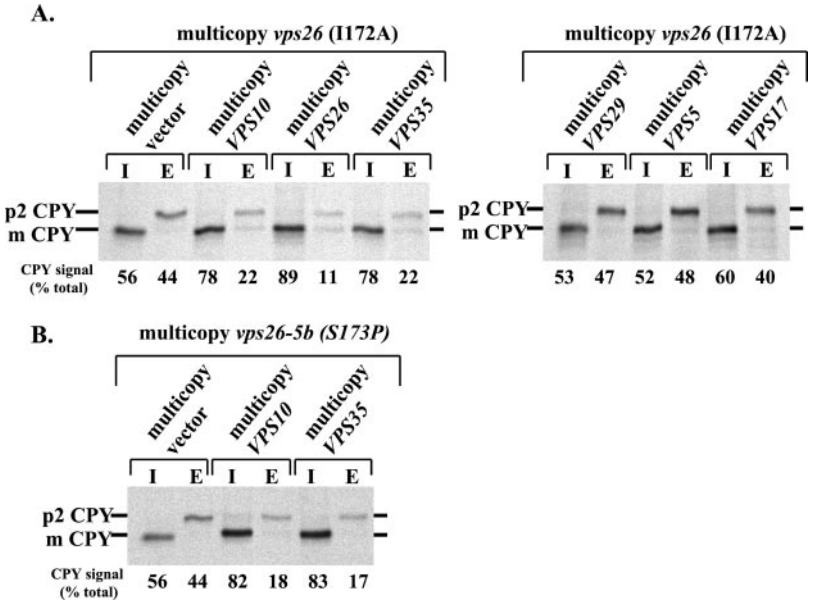

Figure 7. (A) *VPS10* and *VPS35* suppress the dominant negative *vps26* I172A mutant. Wild-type cells were cotransformed with the *vps26* I172A multicopy plasmid and either empty vector or a plasmid to overexpress a retromer component. CPY sorting assays were performed as described previously with intracellular and extracellular fractions being separated after the chase. The gel bands corresponding to mature (m) CPY and p2 CPY were quantitated with the use of NIH Image software. CPY sorting is expressed as a percentage of the total CPY signal. Overexpression of *VPS10*, *VPS26*, or *VPS35* can suppress the CPY sorting defect imposed by the *vps26* I172A mutant. Overexpression of *VPS29*, *VPS5*, or *VPS17* had no effect. (B) Similarly, *VPS10* and *VPS35* can suppress the *vps26* S173P mutant.

contain Vps5p/Vps17p and Vps35p/Vps29p. This indicates that Vps26p interaction with Vps35p may be more transient or possibly more dynamic.

DISCUSSION

Vps26p is a component of the retromer complex and is required for the correct localization of the CPY sorting receptor Vps10p. Although there has been some recent progress toward a better understanding of the function of Vps35p within retromer (Nothwehr *et al.*, 2000), the role that Vps26p performs is unknown. It seems unlikely that Vps26p is an essential structural component of the complex because it has been previously demonstrated that the remaining four subunits can be cross-linked together in *vps26*Δ cells (Seaman *et al.*, 1998). Therefore, we have investigated the role of Vps26p in retromer function and in endosome-to-Golgi transport in general by the generation of dominant negative

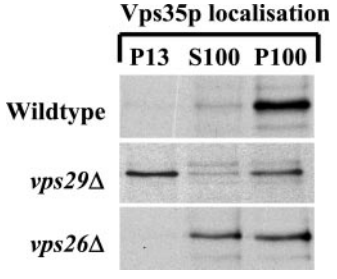

Figure 8. Vps26p is required for the proper membrane association of Vps35p. Wild-type, *vps29*Δ, or *vps26*Δ cells were converted to spheroplasts, labeled, and chased before being lysed in cytosol buffer. Membrane fractions were separated by differential centrifugation. After the final (100,000 × *g*) spin, the supernatant was removed. The fractions were precipitated with TCA, acetone washed, and then solubilized in urea cracking buffer. Vps35p was recovered from the resulting lysates and then subjected to SDS-PAGE. In wild-type cells, Vps35p is peripherally associated with P100 (Golgi, endosome, and small vesicles) membranes. In *vps29*Δ mutants Vps35p follows Vps10p to the vacuolar (P13) membrane fraction, whereas in *vps26*Δ cells, ~50% of the total Vps35p becomes soluble and is detected in the S100 fraction, the remainder is in the P100 fraction.

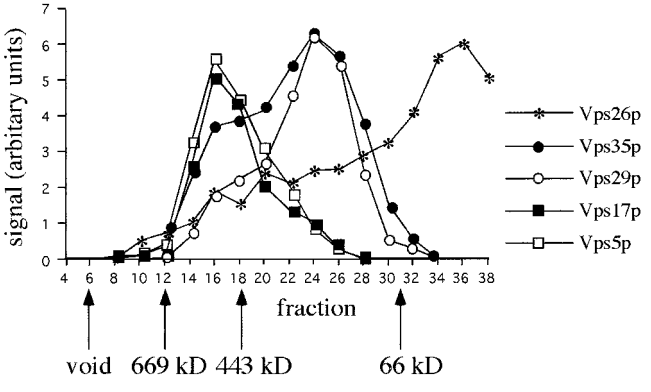

Figure 9. Vps26p interaction with other retromer components may be more transient/dynamic. Wild-type cells (~3000 OD equivalents) were spheroplasted, washed, and then lysed in cytosol buffer. After centrifugation at 13,000 × *g*, a P100 membrane fraction was prepared by centrifugation at 100,000 × *g*. The P100 was then stripped by resuspending into cytosol buffer containing 250 mM NaCl. Membranes were repelleted and the supernatant was then loaded onto a sephacryl S300 column. Fractions were run on a 9% polyacrylamide gel, electrophoretically transferred to nitrocellulose, and then Western blotted with the use of antibodies against retromer components. The resulting autoradiogram was quantitated with the use of NIH Image software. Salt stripping results in Vps26p being displaced from retromer and becoming monomeric, whereas Vps35p remains associated with Vps29p and Vps5p cofractionates with Vps17p.

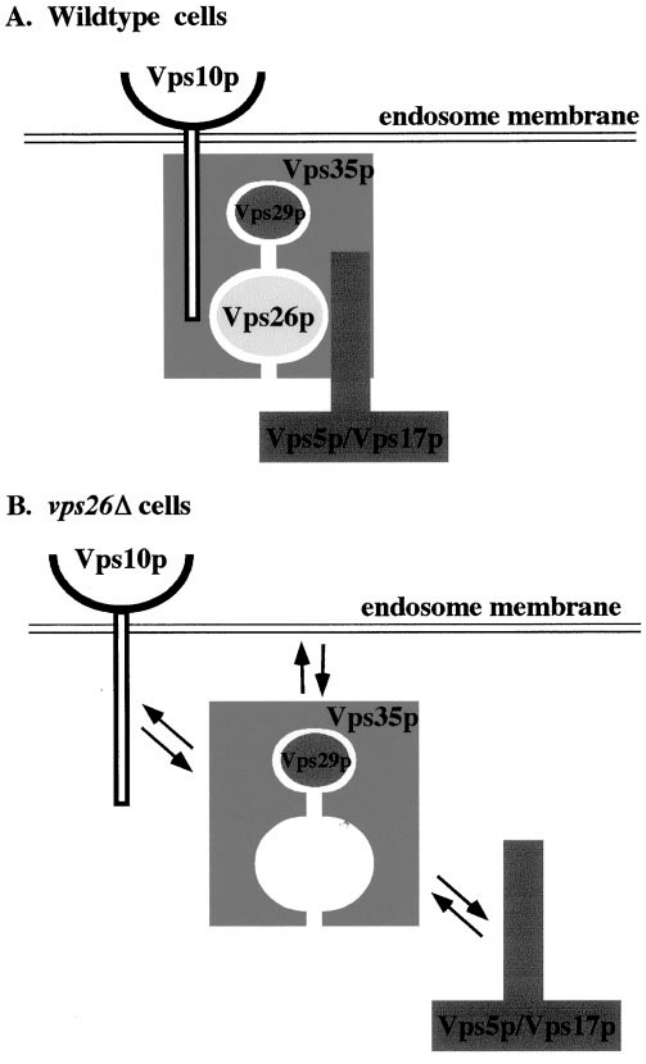

Figure 10. A model for the function of Vps26p within retromer. (A) In wild-type cells, Vps26p interacts with Vps35p to promote/stabilize interactions between Vps35p and Vps5p/Vps17p. Vps26p is also important for maintaining the membrane association of Vps35p and therefore may affect the interactions between Vps35p and cargo molecules such as Vps10p. (B) *vps26*Δ mutants fail to properly localize Vps35p and interactions between Vps35p and Vps5p/Vps17p are significantly weakened.

mutants that interfere with the retrieval of Vps10p from the endosome, resulting in a CPY sorting defect. Additionally, we have investigated the functional importance of different domains of Vps26p by the creation of yeast-mouse hybrid *vps26* molecules.

The dominant negative mutations were generated in a nonbiased way by random PCR mutagenesis, resulting in the isolation of several dominant negative alleles, each with two or three mutations within the region of *VPS26* that was mutated (Table 2). The high frequency of the mutations in the residues 173–174 suggested that mutation of this region was responsible for the dominant negative phenotype. This was confirmed when the S173P mutation was separated from the other mutations present in the *vps26-5* and *vps26-10* alleles, and the effect of the single mutations was tested. Only the S173P mutation conferred the dominant negative phenotype; the other mutations did not result in a dominant negative phenotype (Figure 1A and Table 2).

Furthermore, site-directed mutation of isoleucine at 172 to alanine (I172A) also generated a dominant negative *vps26* allele similar to the S173P mutant. However, the S173A and K174A mutants were not dominant negative (Figure 1B) and were able to fully complement the *vps26*Δ mutant (Figure 2). Clearly, the type of mutation that was generated in the residues 173–174 by the random PCR mutagenesis is as important as the site of the mutation. Substitution of serine 173 to proline would be expected to have a greater impact upon the secondary structure of this region of Vps26p than the change of serine to alanine. The lysine-to-glutamate mutation at 174 results in a change in the charge of this residue. This reversal in the charge at residue 174 may be what is causing the dominant negative phenotype of the K174E mutation and hence a change to alanine may be more tolerated. Indeed, given that the mutations detected in the *vps26* dominant negative alleles were fairly evenly distributed over the protein, it appears that Vps26p may tolerate many mutations without apparent effect upon function.

Another approach to investigating the relative importance of the different regions of Vps26p is by sequence comparisons with homologs and then domain swapping experiments to see whether the highly conserved regions can substitute for one another. The most highly conserved region of Vps26p is the C-terminal third of the protein (Figure 4A). Therefore, a domain swap experiment was performed in which the C-terminal third of Vps26p was replaced with the homologous region of Hβ58, the mouse homolog. This Vps26p-Hβ58 hybrid has been shown previously to fully complement the CPY sorting defect in a *vps26*Δ cell (Bachhawat *et al.*, 1994). We also found this to be the case; however, to our surprise we found that the presence of an HA tag in the N-terminal half of the protein rendered the Vps26p-HA-Hβ58 hybrid completely nonfunctional (Figure 4B). The HA tag alone had no discernible effect upon the function of Vps26p; only the combination of the HA tag with the Hβ58 C terminus resulted in the hybrid protein being nonfunctional. This "intragenic synthetic" phenotype is perhaps suggesting that the C terminus of Vps26p can fold back upon itself so that the C-terminal third and the N-terminal region where the HA tag was placed form a single binding platform for another protein. Other yeast-mouse hybrid Vps26 proteins were found to be nonfunctional and very unstable, indicating that the folding of the hybrid molecules was severely compromised.

Previously, it has been shown that deletion of *VPS26* results in the mislocalization of Vps10p to the vacuole (Seaman *et al.*, 1998). Therefore, it would be reasonable to expect that the dominant negative mutants of Vps26p should also result in the mislocalization of Vps10p to the vacuolar membrane. This was tested by subcellular fractionation of cells expressing the dominant negative S173P *vps26* mutant. We found that the localization of Vps10p is altered (Figure 3A) with ~35% of the Vps10p being detectable in a vacuolar (P13) membrane fraction. Compared with the effect observed in a deletion mutant, the amount of Vps10p present in the vacuolar fraction is fairly modest. These fractionation

data are supported by immunofluorescence observations that showed no discernible redistribution of Vps10p to the vacuole and no gross morphological changes. What effect, if any, is the *vps26* dominant negative mutant having upon Vps10p trafficking? Altered Vps10p trafficking has been shown to result in a proteolytic clipping of Vps10p. This is most apparent in class E *vps* mutant such as *vps4* and *vps27* (Cereghino *et al.*, 1995; Piper *et al.*, 1996). When cells expressing the dominant negative *vps26* mutant were pulse/chased to investigate Vps10p stability, it was found that Vps10p is indeed less stable as a result of the dominant negative mutant expression. Compared with *vps4* cells, however, the instability of Vps10p is not extreme (Figure 3B). Interestingly, *vps4* mutants exhibit a similar CPY sorting defect to cells expressing the dominant negative *vps26* mutant (Babst *et al.*, 1997). Both mutants missort and secrete ~50% of the CPY. It seems unlikely therefore that the instability of Vps10p in a *vps26* dominant negative mutant can be the sole cause for the CPY sorting defect. One possibility is that Vps26p is required for the retrieval of a protein(s) other than Vps10p, which is necessary for the forward transport of CPY to the vacuole. A candidate for this protein would be the SNARE protein, which mediates fusion of Golgi vesicles with the endosome and would have to be recycled back to the Golgi for continued efficient forward transport of CPY. We have attempted to identify this other protein(s) by screening for suppressors of the *vps26* dominant negative mutants, but so far these studies have failed to yield any candidates.

Any effects of the *vps26* dominant negative mutant upon Vps10p localization and stability are likely to be a symptom of a defect in retromer function. So how do the *vps26* dominant negative mutants affect retromer function? One obvious possibility is that the dominant negative mutants are unable to interact with other members of the complex. This was tested by a series of native immunoprecipitation experiments. First, the interactions that Vps26p is able to undergo with other retromer components were examined. Antibodies against Vps26p were able to coimmunoprecipitate all four of the other retromer components (Figure 5A). Immunoprecipitations from extracts prepared from deletion mutants revealed that Vps26p can directly interact with Vps35p. Interactions between Vps26p and other retromer components may be indirect because deletion of Vps35p ablates all the interactions. Previous observations have indicated that Vps26p does not play an essential structural role in retromer assembly (Seaman *et al.*, 1998). This was borne out in the experiment shown in Figure 5B. Immunoprecipitation of Vps5p from *vps26*Δ extracts indicated that the interaction between Vps5p/Vps17p and Vps35p is facilitated by Vps26p but Vps29p is absolutely required for this interaction.

The immunoprecipitation assay was applied to test whether the mutant *vps26* alleles and the Vps26p-Hβ58 yeast-mouse hybrid proteins were able to interact with other members of retromer. Only the Vps26p-HA-Hβ58 failed to fully interact with all other members of the retromer complex with significantly less Vps5p/Vps17p coimmunoprecipitating with Vps26p-HA-Hβ58 (Figure 5C). In this respect, the Vps26p-HA-Hβ58 hybrid protein is similar to a *vps26*Δ mutant in Figure 5B because of the effect upon the interaction between Vps35p/Vps29p and Vps5p/Vps17p. These data may also indicate that the Vps26p-HA-Hβ58 hybrid molecule is unable to interact with the Vps5p/Vps17p subcomplex, although it should be noted that from the native immunoprecipitations of extracts from *vps35*Δ or *vps29*Δ cells, we have no evidence for a direct interaction between Vps26p and Vps5p/Vps17p. Only when Vps35p is present can Vps26p coimmunoprecipitate Vps5p/Vps17p.

The importance of the Vps26p/Vps35p interaction is underscored by the fact that in *vps26*Δ*vps29*Δ double mutants, Vps35p is very unstable. The dominant negative *vps26* alleles can rescue Vps35p stability but the Vps26p-HA-Hβ58 hybrid is unable to perform this function (Figure 6). This indicates that the Vps26p-HA-Hβ58 hybrid is actually unable to properly interact with Vps35p. Clearly, this observation contrasts with the data from the native immunoprecipitation experiment in Figure 5c. One possibility is that Vps29p aids the interaction between Vps35p and Vps26p. This was hinted at in Figure 5a where the amount of Vps35p that could coimmunoprecipitate with Vps26p was slightly reduced in extracts from *vps29* mutants. In the native immunoprecipitation experiment in Figure 5c, Vps29p would have been present and therefore the interaction between the Vps26p-HA-Hβ58 hybrid and Vps35p could be stabilized. Recently, Renfrew-Haft *et al*. (2000) have mapped the interactions that mammalian VPS35 undergoes with the other mammalian retromer components. Their observations have led to suggestions that VPS35 is a binding "raft" for several proteins, one of which is SNX1, the mammalian homolog of Vps5p. We therefore suggest that one of the functions that Vps26p performs is to promote or facilitate the interaction between Vps35p and Vps5p/Vps17p. The interaction between Vps26p and Vps35p that we report here seems to be somewhat less strong than the interaction between Vps35p and Vps29p. Although the interaction between Vps35p and Vps29p is maintained after salt stripping P100 membranes, Vps26p does not significantly cofractionate with the Vps35p/Vps29p subcomplex (Figure 9). In fact after salt stripping, Vps26p is rendered mostly monomeric. This then suggests that the Vps26p/Vps35p interaction may be more transient and/or more dynamic in vivo.

Can Vps26p do more than promote interactions between Vps35p and Vps5p/Vps17p? This question is prompted by the observation that deletion of *VPS26* results in some Vps35p becoming cytosolic (Figure 8). This phenotype is unique to *vps26*Δ cells because it is not seen in *vps29*Δ cells, *vps5*Δ cells, or various double mutant combinations. This suggests that perhaps Vps26p contributes to the membrane association of Vps35p. Clearly, this observation could have implications for the interactions between Vps35p and cargo such as Vps10p. The *vps26* dominant negative alleles (S173P and I172A) could be suppressed by overexpression of *VPS35* and *VPS10* (Figure 7). Although suppression of the CPY sorting defect in cells expressing the *vps26* dominant negative mutants by overexpression of *VPS10* could be accounted for by simply increasing the amount of Vps10p available to bind CPY in the late-Golgi, the suppression of *vps26* mutants by overexpression of *VPS35* suggests that increasing the amount of Vps35p present will improve the retrieval of Vps10p. Given the recent findings by Nothwehr *et al.* (1999, 2000) that Vps35p interacts with cargo molecules such as Vps10p, one possibility is that Vps26p also promotes interactions between cargo and Vps35p. It could do this

either by stimulating Vps35p to interact with cargo, or Vps26p could contribute to the cargo binding site of retromer. These two possibilities are impossible to distinguish at present. The schematic presented in Figure 10 shows how the interaction between Vps26p and Vps35p is required to stabilize and/or facilitate the interactions between Vps35p and cargo such as Vps10p and the structural components Vps5p/Vps17p. In the absence of Vps26p, Vps35p becomes partially soluble and therefore is unable to tightly interact with Vps10p (and other cargo), and interactions between Vps35p and Vps5p/Vps17p are also affected. Alternatively, Vps26p binding to Vps35p may simply activate a membrane binding site within Vps35p itself and hence promote the stable interaction between Vps35p and the membrane. In this context, Vps26p could be a vital regulatory factor in the cycling of Vps35p on and off the membrane. In its role as part of a vesicle coat that drives endosome-to-Golgi retrieval of Vps10p, Vps35p membrane association would be expected to be highly dynamic and coupled to rounds of vesicle formation and subsequent vesicle uncoating.

There is much yet to be learned about how the retromer complex functions and directs retrieval from the endosome to the Golgi. By dissecting the complex and analyzing the function(s) of its constituent components, we and others have started to shed some light upon this process. Because the retromer complex is conserved from yeast to human, we can hopefully apply what we have discovered in a simple eukaryote such as yeast to a more complex organism like human.

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