Vps10p Cycles between the Late-Golgi and Prevacuolar Compartments in Its Function as the Sorting Receptor for Multiple Yeast Vacuolar Hydrolases

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Abstract. *VPS10* (Vacuolar Protein Sorting) encodes a large type I transmembrane protein (Vps10p), involved in the sorting of the soluble vacuolar hydrolase carboxypeptidase Y (CPY) to the *Saccharomyces cerevisiae* lysosome-like vacuole. Cells lacking Vps10p missorted greater than 90% CPY and 50% of another vacuolar hydrolase, PrA, to the cell surface. In vitro equilibrium binding studies established that the 1,380–amino acid lumenal domain of Vps10p binds CPY precursor in a 1:1 stoichiometry, further supporting the assignment of Vps10p as the CPY sorting receptor.

Vps10p has been immunolocalized to the late-Golgi compartment where CPY is sorted away from the secretory pathway. Vps10p is synthesized at a rate 20-fold lower than that of its ligand CPY, which in light of the 1:1 binding stoichiometry, requires that Vps10p must recycle and perform multiple rounds of CPY sorting. The 164–amino acid Vps10p cytosolic domain is involved in receptor trafficking, as deletion of this domain resulted in delivery of the mutant Vps10p to the vacuole, the default destination for membrane proteins in yeast. A tyrosine-based signal ($YSSL_{80}$) within the cytosolic domain enables Vps10p to cycle between the late-Golgi and prevacuolar/endosomal compartments. This tyrosine-based signal is homologous to the recycling signal of the mammalian mannose-6-phosphate receptor. A second yeast gene, *VTH2*, encodes a protein highly homologous to Vps10p which, when overproduced, is capable of suppressing the CPY and PrA missorting defects of a *vps10Δ* strain. These results indicate that a family of related receptors act to target soluble hydrolases to the vacuole.

GENETIC screens have identified a large number of Vacuolar Protein Sorting (VPS)[1] genes involved in the sorting and delivery of carboxypeptidase Y (CPY) to the vacuole (Jones, 1977; Banta et al., 1988; Robinson et al., 1988; Rothman et al., 1989; Raymond et al., 1992). Recently, it has become clear (Stack et al., 1995) that these genes define a post-Golgi protein sorting pathway very similar to what has been described for lysosomal hydrolases in mammalian cells (Kornfeld, 1992). The targeting of mammalian lysosomal proteins involves the addition of a mannose-6-phosphate moiety to soluble hydrolases following their translocation into the endoplasmic reticulum (Kornfeld, 1992). This sorting signal is recognized by the cation-independent mannose-6-phosphate/IGF II receptor (M6PR), which binds newly synthesized proteins in the Golgi and delivers them to the prelysosomal/endosomal compartment. Here the protein dissociates from the receptor in the lower pH environment of the endosome and is subsequently delivered to the lysosome. The receptor is then recycled to the *trans*-Golgi network (TGN) or plasma membrane to sort additional ligand.

In the yeast *Saccharomyces cerevisiae* the vacuolar hydrolase CPY is sorted away from secreted proteins in a late-Golgi compartment, which is likely analogous to the mammalian TGN (Graham and Emr, 1991; Wilsbach and Payne, 1993; Nothwehr et al., 1995; Stack et al., 1995). There is growing evidence that *S. cerevisiae* also has a post-Golgi, prevacuolar compartment analogous to a mammalian prelysosome/late endosome (Raymond et al., 1992; Vida et al., 1993; Piper et al., 1995; Stack et al., 1995). In contrast to the specific recognition of mannose-6-phosphate by the M6PR, yeast vacuolar hydrolases are sorted via a signal within their propeptides (Valls et al., 1987; Johnson et al., 1987; Klionsky et al., 1988). These propeptides are proteolytically cleaved once the hydrolase precursors reach the vacuole (Stevens et al., 1982; Jones, 1991). The best characterized of these sorting signals is

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; M6PR, mannose-6-phosphate/IGF II receptor; mCPY, mature CPY; mPrA, mature PrA; PrA, proteinase A; proCPY, CPY precursor; proPrA, PrA precursor; VPS, vacuolar protein sorting.

within the propeptide of CPY, and is minimally defined by the amino acids QRPL (Valls et al., 1990). Evidence that the sorting of yeast vacuolar hydrolases is receptor mediated is provided by the observations that the sorting capacity of yeast can be saturated by overexpression of either CPY (Stevens et al., 1986) or proteinase A (PrA; Rothman et al., 1986).

The identification and characterization of the CPY receptor is important for several reasons. An analysis of the binding association between the receptor and CPY might reveal in vivo conditions important in regulating binding and dissociation of ligand. Secondly, the receptor is likely to recycle, as does the M6PR, and an investigation of the recycling signals on the receptor should provide important insights into membrane trafficking in yeast. Finally, an analysis of the sorting receptor in *vps* mutant strains should help reveal the molecular basis for the CPY sorting defect in those strains.

Several lines of investigation have indicated that the *VPS10* gene is likely to encode the CPY sorting receptor. The yeast genome sequencing project identified *PEP1/VPS10* as a gene on chromosome II predicted to encode a 178-kD type I transmembrane protein of 1579 residues (van Dyck et al., 1992). The predicted protein, Vps10p, contains a signal sequence at its amino terminus, a large lumenal domain of 1,380 amino acids, a 17–amino acid transmembrane domain and a carboxy-terminal domain of 164 amino acids. Of the many *VPS* genes sequenced, *VPS10* was the first gene identified to encode a transmembrane protein and therefore was a likely candidate for the CPY receptor (Marcusson et al., 1994). These investigators found that Vps10p could be cross-linked to proCPY, but not to a sorting defective mutant form of proCPY. Additional support that Vps10p is the CPY sorting receptor comes from phenotypic studies indicating that *vps10Δ* cells secrete >90% of newly synthesized CPY yet *vps10Δ* cells exhibit normal vacuolar morphology (Banta et al., 1988; Raymond et al., 1992; Marcusson et al., 1994). Because normal vacuole membranes are assembled in *vps10Δ* cells, overall membrane traffic to the vacuole appears to be unaffected. A recent analysis of Vps10p has found the cytosolic domain to influence the membrane trafficking of the protein, and Vps10p lacking this domain is delivered to the vacuole (Cereghino et al., 1995).

Here we report that the lumenal domain of Vps10p binds proCPY stoichiometrically in vitro. In contrast to a previous report (Marcusson et al., 1994), we found that *vps10Δ* cells missort PrA as well as CPY, indicating that Vps10p sorts at least two vacuolar hydrolases. The 1:1 stoichiometry of ligand binding, taken together with the expression levels of receptor and ligand, indicate that Vps10p must recycle to sort the excess of newly synthesized CPY. Vps10p cycles between the late-Golgi and prevacuolar compartments and this recycling is dependent on a tyrosine-based signal within its cytosolic domain. This signal is similar to the recycling signal in the cytosolic domain of the M6PR. Finally, *VTH2* (Vps Ten Homologue) encodes a homologue of Vps10p that, when overexpressed, suppresses the missorting phenotype of *vps10Δ* cells. This suggests that a family of receptors participate to varying degrees in the sorting of soluble hydrolases to the yeast vacuole.

Materials and Methods

Strains, Media, and Microbiological Techniques

Yeast strains used in this study are listed in Table I. Strains were constructed by standard genetic techniques and grown at 30°C in rich media (1% yeast extract, 1% peptone, 2% dextrose; YEPD) or standard minimal medium (SD) with appropriate supplements as described by Sherman et al. (1986). Strain TSY108 was constructed by transforming SF838-5A (*MATα ura3-52, leu2-3,112 ade6*) with linearized plasmid pCAV3, which contains both the *LEU2* and the CPY encoding gene, *PRC1*. pCAV3 was linearized with either XbaI (cleaves in *PRC1*) or ClaI (cleaves in *LEU2*) and the mixture transformed to direct integration of the plasmid borne *PRC1* to both the *LEU2* and *PRC1* loci. Leu$^+$ transformants were selected and screened by CPY overlay blot (Roberts et al., 1991; Piper et al., 1994) to identify colonies overproducing and therefore secreting CPY. TSY108 overproduced CPY ~6–8-fold of which 40% was secreted.

DNA manipulations and DNA-mediated transformation of *E. coli* strains MC1061 and CJ236 were performed by routine procedures.

Materials

Enzymes used in DNA manipulations were from New England Biolabs (Beverly, MA), Boehringer Mannheim Biochemicals (Indianapolis, IN), Bethesda Research Laboratories (Gaithersburg, MD), or U.S. Biochemicals (Cleveland, OH). Goat anti–rabbit and goat anti–mouse alkaline phosphatase conjugates used for Western blots and CPY overlay blots were purchased from Promega (Madison, WI). The ECL kit from Amersham Corp. (Arlington Heights, IL) was used in conjunction with HRP-conjugated antibodies for the development of immunoblots. Secondary antibodies used for indirect immunofluorescence experiments (all cross-species adsorbed) were purchased from Jackson Immunoresearch Labs Inc. (West Grove, PA). Fixed *S. aureus* cells (IgGsorb) were obtained from The Enzyme Center (Malden, MA). ^{35}S-Express label was from New England Nuclear (Boston, MA). Oxalyticase was from Enzogenetics (Corvallis, OR). All other reagents were purchased from Sigma Chem. Co. (St. Louis, MO).

Table I. Yeast Strains Used in This Study

Strain	Genotype	Parent strain
SF838-9D‡	*MATα ura3-52 leu2-3,112 his4-519 ade6 pep4-3*	
RPY10	*MATα ura3-52 leu2-3,112 his4-519 ade6*	SF838-9D
AACY10	*vps10Δ::URA3 pep4-3*	SF838-9D
AACY29	*vps10–10* pep4-3*	SF838-9D
AACY9	*vps10Δ::URA3*	RPY10
AACY11	*vps10–80**	RPY10
AACY12	*vps10–100**	RPY10
AACY13	*vps10–85**	RPY10
AACY15	*vps10–Y_{77}A*	RPY10
AACY16	*vps10–10**	RPY10
AACY17	*vps10–FYVF$_{44}$AAAA*	RPY10
AACY19	*vps10–90**	RPY10
AACY20	*vps10–75**	RPY10
AACY21	*vps10–L_{80}A I_{81}A*	RPY10
AACY22	*vps10–Δ(77-85)*	RPY10
AACY23	*vps10–F_{69}A*	RPY10
AACY24	*vps10–F_{85}A*	RPY10
AACY25	*vps10–L_{80}A*	RPY10
AACY26	*vps10–I_{81}A*	RPY10
AACY27	*vps10–F_{106}A*	RPY10
AACY28	*pho8Δ::LEU2*	RPY10
AACY40	*vps10–Y_{77}A F_{106}A*	RPY10
AACY50	*VPS10::VTH2*	RPY10
SEY6210§	*MATa ura3-52 leu2-3,112 his3-Δ200 trp1-901 lys2-801 suc2-D9*	
AACY30	*vps10Δ::URA3*	SEY6210

‡Rothman et al. (1989).
§Robinson et al. (1988).
Strains AACY9, AACY50, and AACY11 through AACY28 are isogenic to RPY10 except at the *VPS10* locus. AACY30 and SEY6210 are isogenic except at the *VPS10* locus.

Subcloning, Mutagenesis, and Disruption of VPS10

The 2 micron (multicopy) plasmid used to overexpress Vps10p was constructed by subcloning the 6.1-kb HpaI–PvuII fragment of *VPS10* into the SmaI site of YEp352 (Hill et al., 1986) to produce pKE50. Flanking SalI and SacI sites in pKE50 permitted the 6.1-kb fragment containing *VPS10* to be inserted into pRS315 (Sikorski and Hieter, 1989) cut with SalI and SacI to generate plasmid pAAC200.

The *vps10Δ::URA3* allele used to disrupt the *VPS10* locus was constructed as follows: pAAC214 contains the 6.1-kb SalI–SacI fragment of *VPS10* inserted into pBluescript II KS$^+$ (Stratagene, La Jolla, CA) cut with SalI and SacI. Site-directed mutagenesis was performed using the bacterial strain CJ236 and the helper phage M13KO7 according to the method of Kunkel et al. (1987) to introduce a BglII site immediately downstream of the *VPS10* initiation codon thereby creating plasmid pAAC215. This plasmid was cut with StuI and a BglII linker (Cat No. 1051, New England Biolabs) inserted to produce pAAC216. pAAC216 was cut with BglII and the intervening 4.8-kb fragment containing the entire *VPS10* open reading frame was removed and replaced with a 1.1-kb BamHI fragment containing the *URA3* gene from plasmid pEF45. The resulting plasmid, pAAC220, was cleaved with SacI and XhoI before transformation into RPY10 and SF838-9D to create yeast strains AACY9 (*vps10Δ::URA3*) and AACY10 (*vps10Δ::URA3 pep4-3*), respectively. A CPY overlay blot (Roberts et al., 1991; Piper et al., 1994) was used to identify Vps$^-$ transformants using the anti-CPY monoclonal antibody (No. 10A5-B5) obtained from Molecular Probes, Inc. (Eugene, OR). Selected transformants were confirmed as *vps10Δ* by Western blot analysis using anti-Vps10p antibodies.

The 1.4-kb PstI–SacI fragment encoding the cytoplasmic domain of *VPS10* was subcloned into pBluescript II SK$^+$ cut with the same enzymes to give plasmid pAAC210. Single stranded DNA derived from pAAC210 was used in oligonucleotide directed mutagenesis to create the substitution mutation $FYVF_{44}$ to $AAAA_{44}$. This mutation was introduced into the context of the full-length *VPS10* to give plasmid pAAC230.

The remaining mutations introduced into the Vps10p cytoplasmic domain were accomplished by PCR amplification. The 0.7-kb PstI–StuI fragment of *VPS10* that encodes the cytoplasmic domain was amplified from wild-type *VPS10* DNA with oligonucleotides in either one-step (for carboxy-terminal truncations) or two-step (for the introduction of mutations internal to the 0.7-kb fragment) amplification steps. The resulting mutant fragments were cleaved with PstI and StuI before insertion into pAAC200 cut with the same enzymes. The Vps10p–Vth2p fusion was created in a similar manner with the two-step amplification procedure using (*a*) a fragment of *VPS10* from the PstI site to the end of the sequence encoding the Vps10p transmembrane domain and (*b*) an amplified fragment of the Vth2p domain with the 5′ oligonucleotide also containing sequence to the Vps10p transmembrane domain. The amplification of these two fragments using flanking DNA primers produced the intended fusion. The mutagenized region of all of these plasmids was confirmed by DNA sequencing using the method of Sanger et al. (1977).

To integrate the *vps10* mutant alleles into a wild-type *VPS10* yeast strain, the plasmids containing the mutant *vps10* alleles were cleaved with SacI and SalI to release the *vps10* containing fragment, the fragment was cotransformed with pRS315 (*LEU2*) into yeast strain AACY9 (*vps10Δ::URA3*), and Leu$^+$ transformants selected (Rothstein, 1991). Transformants were replica plated onto 5-fluoroorotic acid to select for loss of the *URA3* marker (Boeke et al., 1984) at the *VPS10* locus, which indicated that the mutant *vps10* allele had integrated at the correct chromosomal site. The resulting *vps10* strains are listed in Table I.

To create the *GAL1-vps10-10** allele the plasmid containing *vps10-10** was cleaved with SacI and BglII which cleaves ~140 bp upstream of the *VPS10* initiation codon. The resulting 5-kb fragment was ligated with a 0.8-kb SalI–BamH1 fragment containing the *GAL1* promoter and inserted into the 2 micron (multi-copy) plasmid YEp351 cleaved with SalI and SacI to give plasmid pAAC241.

Cloning of VTH2

VTH2 was identified from the yeast genome database as a large open reading frame (Genbank accession numbers Z34098, Z46921) encoding a predicted protein with extensive homology to Vps10p. Two segments of sequence flanking *VTH2* were amplified by PCR such that they both contained an EcoRI site at the termini closest to *VTH2*. The two fragments were ligated together at the EcoRI site and cloned into the plasmid pRS316 (Sikorski and Hieter, 1989). The resulting plasmid was linearized with EcoRI and transformed into RPY10 to rescue the wild-type *VTH2* allele. Plasmids were rescued from the Ura$^+$ transformants and analyzed to confirm the presence of *VTH2*. The *VTH2* containing fragment was cloned into the 2 micron multi-copy vector YEp351. This gap rescue approach was taken to avoid any potential mutations that may be introduced while amplifying a large 7-kb fragment by PCR.

Vps10p Antibodies

To generate rabbit polyclonal antibodies directed against Vps10p, the following DNA constructs were prepared. The 1.17-kb DraI–SpeI fragment of the *VPS10* gene (amino acids 306-696) was inserted into the SmaI–XbaI sites of pEXP2-S to yield pAAC208. A second bacterial expression plasmid for Vps10p was made by subcloning the DraI–SpeI fragment of *VPS10* into the plasmid pMAL-c2 (New England Biolabs) to produce pAAC209. Induction of *E. coli* carrying plasmid pAAC208 with IPTG produced a fusion protein that was purified and used to inject New Zealand white rabbits for antibody elicitation (Roberts et al., 1991). Antiserum recognizing Vps10p was affinity-purified against protein produced from pAAC209 using the method of Roberts et al. (1991).

Immunoprecipitations of ^{35}S-labeled Proteins

Secretion of newly synthesized CPY was quantified by immunoprecipitation as previously described (Piper et al., 1994). Briefly, yeast cultures were grown overnight in selective synthetic media without methionine to OD_{600} = 1, centrifuged, and resuspended at OD_{600} = 1 in SD-Met/50 mM potassium phosphate, pH 5.7, containing 2 mg/ml BSA. 0.5 OD_{600} of cells were pulse-labeled for 10 min with 100 μCi ^{35}S-Express label and chased for 30 min upon addition of unlabeled methionine and cysteine to a final concentration of 100 μg/ml. The chase was terminated by the addition of sodium azide to 10 mM at 0°C. Cells were separated from the culture media by centrifugation and then spheroplasted. CPY was immunoprecipitated from the resulting intracellular (I; spheroplasts) and extracellular (E; media supernatant) fractions and analyzed by SDS-PAGE and fluorography (Stevens et al., 1986). Vps10p was immunoprecipitated and analyzed in the same manner as CPY except that labeling was performed in nonbuffered SD media and no extracellular fraction was collected. Radioactivity was quantified using an AMBIS Radioanalytic Imaging System (Ambis, San Diego, CA).

Fluorescence Microscopy and Image Processing

Indirect immunofluorescence microscopy was performed as described previously (Roberts et al., 1991) using affinity-purified rabbit polyclonal anti-Vps10p antibodies described above and mouse polyclonal anti-alkaline phosphatase antibodies adsorbed against AACY28 (*pho8Δ*) cells to localize the fusion protein, A-ALP (Nothwehr et al., 1993). Cells were examined using an Axioplan fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY) and photographed with T-MAX 400 film.

Immunofluorescence micrographs and autoradiograms were digitized with a Relisys flatbed scanner (Reli 4816: Milpitas, CA). Images within the same experiment were processed with Adobe Photoshop™ using identical settings and printed on a Tectronics Phaser 440 dye-sublimation printer.

In Vitro Binding Assay

A BamHI site and six-His tag were introduced immediately following the predicted signal sequence cleavage site of Vps10p. In addition, a BamHI site was inserted into the *VPS10* sequence at the position encoding the end of the predicted luminal domain (bp +4185). This 4.2-kb BamHI fragment was inserted in-frame with the *PHO1* signal sequence contained within the plasmid pHIL-S1 (Invitrogen, San Diego, CA) to create pAAC231. After signal sequence cleavage, the soluble form of Vps10p encoded by pAAC231 is predicted to have the following amino-terminal sequence REFPSHHHHHHFT while the predicted amino terminus of native Vps10p is EEFT. pAAC231 was cleaved with BglII and integrated into *Pichia pastoris* strain GS115 to create strain AACY6. Genetic manipulations, growth, and expression of Vps10p by *Pichia pastoris* was performed as described by Invitrogen. After growth and induction of Vps10p expression in BMGY media, the culture supernatant was dialyzed against 50 mM KPO_4, pH 8.0, 50 mM NaCl to raise the supernatant pH to approximately pH 7.5. The supernatant was then loaded onto a Ni^{2+}-NTA-Agarose (Qiagen, Chatsworth, CA) column and washed with 50 mM KPO_4, pH 8.0, 250 mM NaCl before elution with 50 mM KPO_4, pH 8.0, 50 mM NaCl, 200 mM imidazole. The eluted material was dialyzed against 50 mM

Hepes, pH 7.25, 50 mM NaCl before concentration in a Centriprep 30 (Amicon, Beverly, MA). proCPY was purified from the culture medium of *S. cerevisiae* cells overexpressing CPY as described (Winther and Sorensen, 1991). Mature CPY was purchased from Sigma Chem. Co.

Fluorescence based binding assays were performed at 22°C in 50 mM Hepes, pH 7.25, 50 mM NaCl buffer using a SLMOAMINCO spectrofluorometer, model MC320 (SLM Instruments Inc., Urbana, IL) with excitation at 300 nm and emission monitored at 327 nm.

Results

Vps10p Is the CPY Sorting Receptor and Binds proCPY Stoichiometrically

Overexpression of CPY in wild-type yeast cells has been found to result in secretion of the Golgi-modified p2 precursor form (proCPY, Stevens et al., 1986), suggesting that a saturable receptor is responsible for the sorting of CPY to the vacuole. Given the premise of a saturable receptor for the sorting of vacuolar hydrolases, we reasoned that overexpression of such a receptor should suppress the CPY secretion phenotype resulting from CPY overproduction. To investigate this, a strain was constructed that stably overproduced CPY by integrating several copies of *PRC1* at different loci of yeast strain SF838-5A. The resulting strain, TSY108, overproduced CPY approximately six- to eightfold and secreted ~40% of the newly synthesized protein as proCPY (Fig. 1, lanes *5* and *6*), whereas the parent strain SF838-5A correctly sorted CPY (Fig. 1, lanes *1* and *2*). When *VPS10* was overexpressed in TSY108, the level of CPY secretion was reduced to <10% (Fig. 1, lanes *7* and *8*). This suppression of CPY secretion by Vps10p overproduction supports the assignment (Marcusson et al., 1994) of Vps10p as the CPY sorting receptor.

The Golgi-modified precursor proCPY has been found to cross-link to Vps10p using a membrane permeable cross-linker added to yeast spheroplast lysates (Marcusson et al., 1994). To establish more directly that Vps10p is the CPY receptor and to determine the binding stoichiometry, we developed a fluorescence assay to monitor the binding of proCPY to Vps10p. The receptor was produced by expressing the lumenal domain of Vps10p as a secreted protein in the methylotrophic yeast *Pichia pastoris.* To assist the purification of the receptor, a six-His tag was engineered immediately following the predicted signal sequence cleavage site. This DNA construct was integrated into *P. pastoris*, and, upon induction with methanol, a protein of the expected molecular mass (~160 kD) was recovered in the culture supernatant that reacted with anti-Vps10p antibodies. This soluble form of Vps10p from *P. pastoris* and proCPY isolated from *S. cerevisiae* were purified and analyzed by SDS-PAGE to estimate purity (Fig. 2 *C*).

The Vps10p lumenal domain was used with proCPY in a fluorescence equilibrium binding assay, and the data analyzed by a variation of Job's method of analysis (Gil and

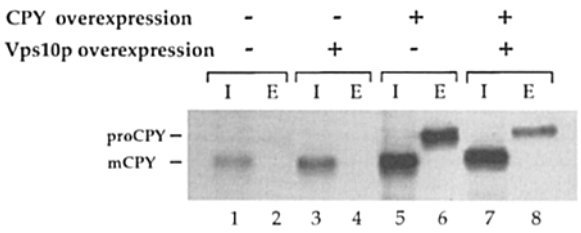


Figure 1. Suppression of CPY secretion by overexpression of Vps10p. Strain SF838-5A (*PRC1*; lanes *1–4*) and TSY108 (multicopy *PRC1*; lanes *5–8*) were transformed with either YEp352 (vector; lanes *1–2* and *5–6*) or pKE50 (multicopy *VPS10*; lanes *3–4* and *7–8*), radiolabeled for 10 min, and chased for 30 min before harvest. The cells and growth media were treated as described and CPY immunoprecipitated from either the intracellular (*I*) or extracellular (*E*) fractions before SDS-PAGE analysis and fluorography. "+" indicates overexpression of Vps10p and/or CPY whereas "−" indicates no overexpression. mCPY refers to the mature form of CPY whereas proCPY refers to the precursor form of CPY.

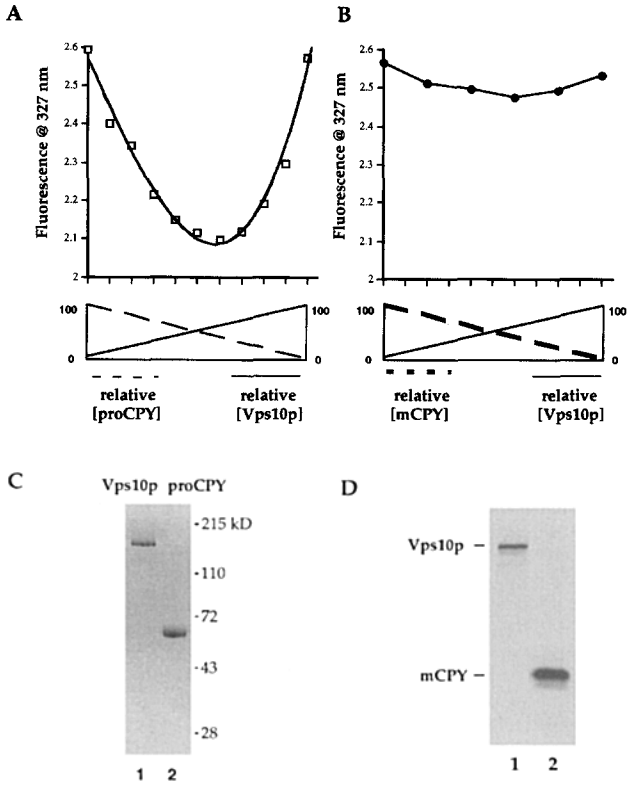


Figure 2. Binding of proCPY by the lumenal domain of Vps10p. (*A*) Graphical representation of the fluorescence quenching effect resulting from Vps10p binding proCPY. Different amounts of a solution, containing either proCPY or Vps10p, were added to a total volume of 1.5 ml. The solution was excited at 300 nm and the emission signal at 327 nm was plotted against the varying concentrations of both proCPY and Vps10p. (*B*) Graphical representation of the fluorescence quenching effect resulting from combining Vps10p and mCPY as described above in *A*. (*C*) Purified preparations of the lumenal domain of Vps10p (lane *1*) and proCPY (lane *2*) were analyzed by SDS-PAGE and staining with Coomassie brillant blue R250. (*D*) Quantitative immunoprecipitation of Vps10p and CPY. Strain RPY10 (*VPS10*) was radiolabeled for 10 min and chased for 30 min before harvest. The intracellular fraction was immunoprecipitated with saturating levels of anti-Vps10p antibodies (lane *1*) or anti-CPY (lane *2*) antibodies before SDS-PAGE analysis and fluorography. The amount of each protein immunoprecipitated was quantified and the Vps10p:CPY synthesis ratio obtained by dividing the counts per minute by the total number of methionine and cysteine residues in Vps10p and mCPY and taking account of the proportion of [S^{35}]cysteine and methionine within ^{35}S-Express label.

Oliveira, 1990; Drago, 1992; Nowick et al., 1993). This approach used the fluorescence of tryptophan residues of both Vps10p and proCPY and the change in fluorescence signal intensity upon association of the two proteins. Both proteins were brought to an empirically determined concentration in which the fluorescence emission signal of the two separate solutions was identical. If no interaction occurs between the two proteins, then mixing the two solutions would yield the same fluorescent signal as either solution alone. Mixing different proportions of the two solutions and plotting the fluorescence intensity against the proportion of one component in the composite solution should give a flat line if no association occurs. Such a mixing experiment was performed, and the parabola-like curve (Fig. 2 *A*) obtained indicates that the two proteins indeed interact. In this case, signal quenching occurred upon binding, and the concentration of Vps10p and proCPY that produced the greatest quenching effect corresponds to conditions in which the maximal amount of complex is formed. That concentration was 0.92 μM for proCPY and 1.05 μM for Vps10p, which indicated that the stoichiometry for the interaction between proCPY and Vps10p as 1:1.14, or approximately 1:1.

To control for binding specificity, we determined whether Vps10p would interact with mature CPY, since mature CPY lacks the 91–amino acid propeptide containing the vacuolar targeting signal (Valls et al., 1987, 1990; Johnson et al., 1987). The lack of fluorescence quenching when Vps10p and mCPY were mixed indicates little or no interaction (Fig. 2 *B*). These data indicate that the binding interaction between Vps10p and proCPY is specific, and that the in vitro binding stoichiometry is 1:1. While the physiological conditions of the late-Golgi lumen are unknown, it seems reasonable to infer that the in vivo proCPY:Vps10p binding stoichiometry would also be 1:1.

To examine the possible requirement of Vps10p recycling for the delivery of CPY to the vacuole, we measured the synthesis rates of both proteins. Quantitative immunoprecipitations (Fig. 2 *D*) of radiolabeled receptor (Vps10p) and ligand (CPY) indicated that CPY was synthesized at ~22-fold the rate of Vps10p synthesis. Since the stoichiometry of CPY binding to Vps10p is 1:1, and the ligand (CPY) is produced at over 20-fold the level of receptor, Vps10p must recycle back to the Golgi to initiate multiple rounds of sorting.

Vps10p Is Required for Sorting Both CPY and PrA

Disruption of the *VPS10* gene in yeast (AACY9) resulted in 95% of CPY being missorted to the cell surface (Fig. 3, lanes *3* and *4*). Surprisingly, *vps10Δ* cells also secreted >60% of another soluble vacuolar hydrolase proteinase A (PrA; lanes *11* and *12*), which suggested that Vps10p may be the receptor involved in the sorting of PrA as well as CPY. That Vps10p is required for sorting PrA is in contrast to a previous analysis of *vps10Δ* cells by Marcusson et al. (1994) who found that 90–95% of the CPY was missorted but <10% PrA was secreted by *vps10* cells. To determine whether strain differences contributed to this discrepancy, the *VPS10* locus was disrupted in the parent strain used in the previous study (SEY6210). However, the resulting strain, AACY30, was also found to secrete 93% of CPY and 51% of PrA (Fig. 3 *A*, lanes *7* and *8*, *15* and *16*). The sorting efficiency of a third vacuolar hydrolase, proteinase B, was not significantly altered upon disruption of *VPS10* (data not shown), consistent with the findings of Marcusson et al. (1994).

If Vps10p sorted both CPY and PrA, then overexpression of one ligand may saturate this receptor and result in secretion of the second ligand. In support of this we found that overexpression of PrA did result in the secretion of a small but significant amount of CPY (Fig. 3 *B*), which suggests that PrA and CPY share the same or overlapping binding sites in Vps10p. Overexpression of proteinase B did not affect the sorting efficiency of CPY (data not shown), which, along with the *vps10Δ* data, suggests that Vps10p does not participate in the sorting of PrB.

VTH2 Encodes a Vps10p Homologue Capable of Sorting Both CPY and PrA

Vps10p is responsible for sorting CPY and approximately half of PrA, suggesting that other receptors may be responsible for sorting the remainder of PrA and other soluble vacuolar hydrolases such as proteinase B and aminopeptidase Y (Nishizawa et al., 1994) to the vacuole. We have identified two *VPS10*-like genes, termed *VTH1* and *VTH2* (Vps Ten Homologue), through cloning and database analysis. *VTH1* and *VTH2* differ by only 1 bp in 4.7 kb and are essentially two alleles of the same gene, presumably the result of a large duplication and translocation between the telomeric regions of chromosome IX (*VTH1*) and X (*VTH2*). For simplicity we will refer to them both as *VTH2*. *VTH2* encodes a predicted protein of similar size (1549 residues) and topology to Vps10p. The 68% amino acid identity between the large lumenal domains of Vps10p and Vth2p suggests that Vth2p may be capable of binding/sorting CPY. However, cells lacking Vps10p secrete >95% of the CPY synthesized, which infers that at wild-type levels Vth2p does not participate significantly in the sorting of CPY. If Vth2p is capable of binding CPY, then overexpression of *VTH2* in a *vps10Δ* strain might suppress the secretion of CPY. Fig. 4 shows that overexpression of *VTH2* in a strain disrupted for *VPS10* (AACY9) halved the extent of proCPY secretion to 47%. A similar experiment was performed to assess whether Vth2p could sort PrA. Fig. 4 (lanes *7* and *8*) shows that the PrA sorting defect associated with *vps10Δ* cells was also significantly suppressed by overproduction of Vth2p. These data indicate that Vth2p can serve as a sorting receptor for both CPY and PrA. *VTH1* and *VTH2* were disrupted to determine if wild-type levels of Vth2p contribute to the sorting of PrA but not result in secretion of PrA (data not shown). The disruption of both *VTH1* and *VTH2* in a *vps10Δ* strain did not increase the degree of PrA secretion associated with a disruption of *vps10Δ* alone (data not shown). Therefore, it appears that Vth1p and Vth2p, while capable of sorting CPY and PrA, are likely to also serve as receptors for other vacuolar proteins.

The Vps10p Cytosolic Domain Is Essential for Vps10p Localization and CPY Sorting

The yeast late-Golgi membrane proteins Kex1p, Kex2p, and DPAP A achieve their Golgi localization through in-

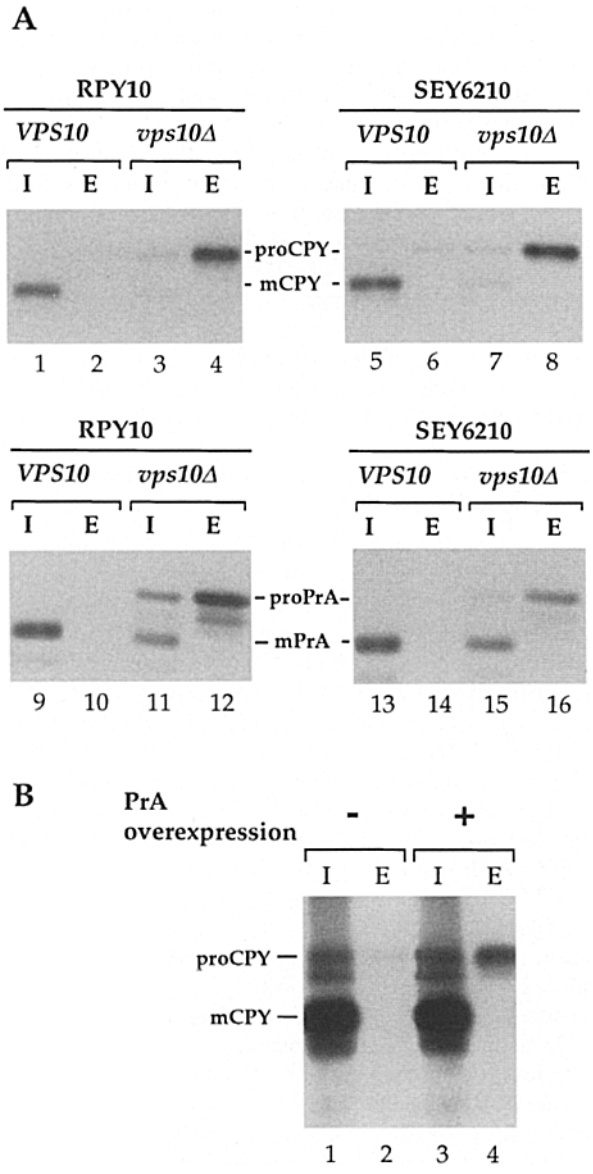


Figure 3. Vps10p is required to sort both CPY and PrA. (*A*) Strains RPY10 and SEY6210 were disrupted at the *VPS10* locus to give strains AACY9 and AACY30, respectively. RPY10 (*VPS10*, lanes *1–2* and *9–10*) and AACY9 (*vps10Δ*, lanes *3–4* and *11–12*), along with SEY6210 (*VPS10*, lanes *5–6* and *13–14*) and AACY30 (*vps10Δ*, lanes *7–8* and *15–16*) cells were radiolabeled for 10 min, and chased for 30 min before immunoprecipitation of CPY (lanes *1–8*) or PrA (lanes *9–16*) from intracellular (*I*) and extracellular (*E*) fractions, and the immunoprecipitated proteins analyzed by SDS-PAGE and fluorography. (*B*) Strain RPY10 (*VPS10*), transformed with either YEp24 (vector) or pPA1 (multi-copy plasmid expressing PrA; Rothman et al., 1986) was radiolabeled, immunoprecipitated with anti-CPY antisera, and analyzed as described above.

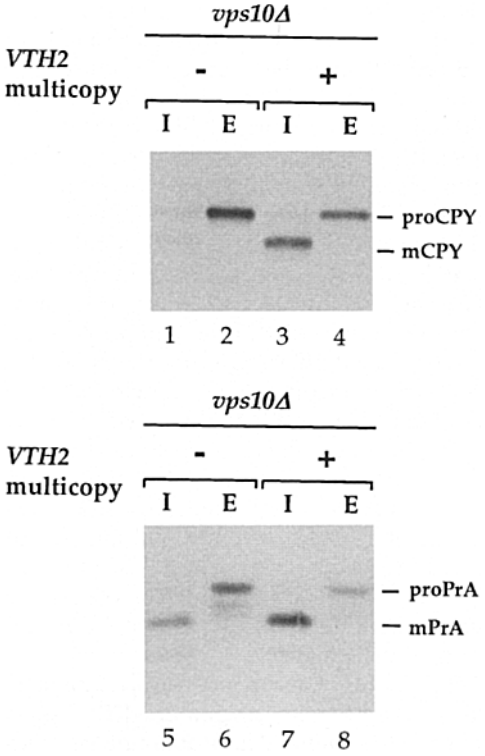


Figure 4. Vth2p is capable of sorting both CPY and PrA. Strain AACY9 (*vps10Δ*) was transformed with either vector (YEp351; lanes *1–2* and *5–6*) or a multicopy plasmid containing *VTH2* (lanes *3–4* and *7–8*). Cells were radiolabeled for 10 min, chased for 30 min before immunoprecipitation of CPY (lanes *1–4*) or PrA (lanes *5–8*) from intracellular (*I*) and extracellular (*E*) fractions, and the immunoprecipitated proteins analyzed by SDS-PAGE and fluorography.

teractions involving their cytosolic domains, and removal of their Golgi retention signals results in the delivery of these proteins to the vacuole where they are subject to vacuolar protease-dependent (*PEP4*) cleavage (Jones et al., 1982; Cooper and Bussey, 1992; Roberts et al., 1992; Wilcox et al., 1992; Nothwehr et al., 1993; Nothwehr and Stevens, 1994). Vps10p is predicted to be a type I membrane protein with a carboxy-terminal, cytosolically exposed domain of 164 amino acids (van Dyck et al., 1992; Marcusson et al., 1994). For clarity we have referred to these residues as 1–164 rather than residues 1416–1579, respectively, where amino acid 1 is immediately adjacent to the transmembrane domain. To investigate whether, like other resident late-Golgi membrane proteins, Vps10p is retained via its cytosolic domain, we deleted this domain and examined the effect on both CPY secretion and Vps10p localization.

A deletion of the 155 carboxy-terminal residues of the Vps10p cytosolic domain was generated by the introduction of a stop codon at codon 10 of the domain (designated as Vps10p-10*). This allele was integrated into the yeast genome to replace the wild-type *VPS10* allele and the mutant cells assayed for CPY secretion. Vps10p-10* containing cells missorted 90–95% of CPY (Fig. 5 *A*, lanes *5* and *6*), similar to the amount secreted by *vps10Δ* cells (Fig. 5 *A*, lanes *3* and *4*). In addition, Vps10p-10* was rapidly proteolytically cleaved to remove an ~15–20-kD fragment in a *PEP4*-dependent manner, indicating delivery of Vps10p-10* to the vacuole (Fig. 5 *B*). These effects resulting from the removal of the Vps10p cytosolic domain are consistent with those reported previously (Cereghino et al., 1995).

To determine whether CPY secretion was a result of mistargeting of Vps10p-10* or of an abrogation of its abil-

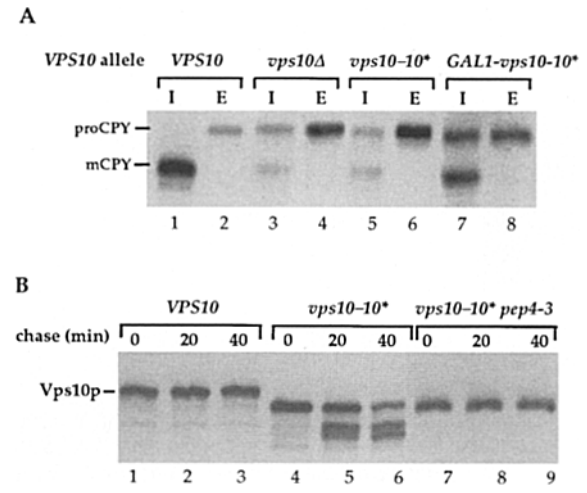


Figure 5. Vps10p lacking its cytosolic domain (*Vps10p-10**) is delivered to the vacuole and fails to sort CPY. (*A*) Strains RPY10 (*VPS10*, lanes *1–2*), AACY9 (*vps10Δ*, lanes *3–4*), AACY16 (*vps10-10**, lanes *5–6*), and AACY9 transformed with pAAC241 (*GAL-vps10-10**, lanes *7–8*) were grown in media containing raffinose (2%) and galactose (2%) as the carbon source before radiolabeling for 10 min and chasing for 30 min. The intracellular (*I*) and extracellular (*E*) fractions were then treated as described in Fig. 1 to immunoprecipitate CPY before analysis by SDS-PAGE and fluorography. (*B*) Strains RPY10 (*VPS10*, lanes *1–3*), AACY16 (*vps10-10**, lanes *4–6*), and AACY29 (*vps10-10* pep4-3*, lanes *7–9*) were radiolabeled for 10 min and chased for the indicated times before harvest. The intracellular fraction was immunoprecipitated with anti-Vps10p antibodies before SDS-PAGE analysis and fluorography.

ity to bind proCPY, we overexpressed the mutant Vps10p-10* and examined the effect on CPY secretion. Cells (*vps10Δ*) overexpressing Vps10p-10* correctly sorted 34% of their CPY to the vacuole compared to only 6% correctly sorted in cells expressing wild-type levels of Vps10p-10* (Fig 5 *A*, lanes *5–8*). This result demonstrates that Vps10p-10* is capable of binding proCPY. Given that Vps10p-10* was delivered to the vacuole and yet was unable to efficiently sort CPY to the vacuole, we hypothesize that recycling of Vps10p back to the Golgi from a post-Golgi/prevacuolar compartment is normally required for efficient sorting.

We performed immunofluorescent studies to immunolocalize wild-type and mutant Vps10p. In wild-type cells, affinity-purified anti-Vps10p antibodies revealed a punctate pattern of ~5–10 small structures per cell, and this signal was specific for Vps10p (Figs. 6, *A* and *B*). Such a pattern has been observed for a number of other proteins localized to the yeast late-Golgi compartment (Redding et al., 1991; Cooper and Bussey, 1992; Roberts et al., 1992; Nothwehr et al., 1993). To confirm this assignment we determined whether Vps10p colocalized with a late-Golgi membrane protein, A-ALP. A-ALP is a fusion protein comprised of the cytoplasmic domain and sorting determinant of DPAP A (Ste13p), a late-Golgi membrane protein, fused to the transmembrane and lumenal domains of alkaline phosphatase (Nothwehr et al., 1993). The A-ALP protein exhibits extensive colocalization with Kex2p (Nothwehr et al., 1993), a late-Golgi membrane protein (Redding et al., 1991; Wilcox et al., 1992). In an indirect immunofluorescence analysis of cells labeled for both Vps10p and A-ALP, morphometric analysis showed that of the cells labeled for both antigens, >70% exhibited colocalization of these antigens (Fig. 6 *A*). Therefore, it is likely that Vps10p binds proCPY in a late-Golgi compartment that coincides or overlaps with the compartment that contains Kex2p and A-ALP.

Vps10p lacking the cytosolic domain was immunolocalized to confirm the conclusion that Vps10p-10* was delivered to the vacuole (Fig. 5 *B*). Fig. 6 *C* shows that Vps10p-10* was indeed localized to the vacuole as judged by the complete overlap between the anti-Vps10p antibody staining and the position of the vacuole as revealed by Nomarski optics, consistent with the observations of Emr and colleagues (Cereghino et al., 1995). Therefore, Vps10p requires the cytosolic domain for Golgi localization and for its CPY sorting function, and, in the absence of this domain Vps10p-10* is delivered rapidly to the vacuole. Therefore, the Vps10p cytosolic domain is likely to contain retrieval signal(s) that acts to return the receptor from the prevacuolar compartment to the Golgi complex.

The Cytosolic Domain of VTH2 Can Substitute for the Homologous Vps10p Domain

An analysis of the Vps10p retrieval signal was aided by a comparison with the homologous domain of Vth2p. An alignment of the Vps10p and Vth2p cytosolic domains showed extensive amino acid identity (52%; Fig. 7 *A*), suggesting that the cytosolic domain of Vth2p may function in a manner similar to that of Vps10p. To test this hypothesis a fusion protein was constructed in which the cytosolic domain of Vth2p was precisely substituted for that of Vps10p. This *VPS10::VTH2* gene fusion was then integrated into the yeast genome at the *VPS10* locus and the resulting yeast strain (AACY50) assayed for CPY sorting function. The Vps10p-Vth2p fusion protein was expressed at levels identical to wild-type Vps10p and was functionally indistinguishable from Vps10p in that yeast cells expressing only Vps10p-Vth2p correctly sorted CPY (Fig. 7 *B*). The functional substitution of the Vth2p cytosolic domain for that of Vps10p, and the high degree of homology between the two, indicates that the sorting/recycling signals are common to both proteins.

A Tyrosine-based Signal in the Cytosolic Domain Is Required for Vps10p Recycling

To identify the recycling signal of Vps10p, a mutagenic analysis of the Vps10p cytosolic domain was performed. It initially involved plasmid-borne copies of *VPS10* transformed into a *vps10Δ* strain, however, the plasmid copy of *VPS10* did not fully complement the *vps10Δ* mutation, and the encoded protein had a shorter half-life than Vps10p encoded from a chromosomal copy of the same gene. Only upon integration of *VPS10* into a *vps10Δ* strain did we obtain complete complementation. The phenotypic discrepancy between an integrated and plasmid-borne *vps10* allele is illustrated in Fig. 8 (lanes *9* and *10* vs *13* and *14*) in which cells containing the integrated allele *vps10-90** secreted 30% CPY, whereas a *vps10Δ* strain carrying the

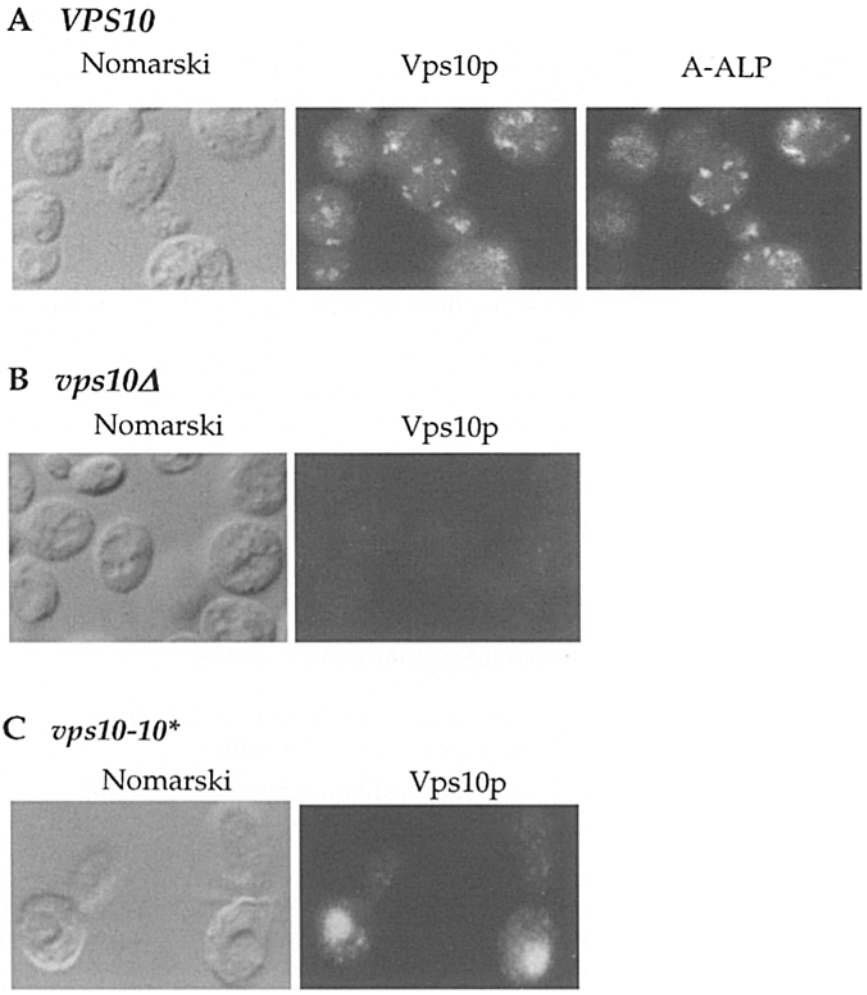


Figure 6. Immunolocalization of Vps10p and Vps10p-10*. (*A*) Strain AACY28 (*VPS10 pho8Δ*) was transformed with pSN55 (A-ALP, Nothwehr et al., 1993), and cells were fixed, spheroplasted, and stained with rabbit antibodies against Vps10p and with mouse polyclonal antibodies against alkaline phosphatase. The cells were viewed by Nomarski optics and epifluorescence through filter sets specific for fluorescein (anti-Vps10p antibodies) and Texas red (anti-ALP antibodies). (*B*) Strain AACY9 (*vps10Δ*) was treated as described above and stained with anti-Vps10p antibody before viewing with Nomarski optics and epifluorescence through a filter set specific for fluorescein. (*C*) Strain AACY29 (*vps10-10* pep4-3*) was fixed, spheroplasted, and stained with anti-Vps10p antibodies before viewing with Nomarski optics or epifluorescence through a filter set specific for fluorescein.

plasmid borne *vps10-90** allele (*CEN-90**) secreted 52% of the CPY synthesized. Therefore, as opposed to an earlier analysis that expressed *VPS10* from plasmids (Cereghino et al., 1995), all of the mutations described in this paper were integrated into the *VPS10* locus before analysis.

A series of deletions from the carboxy terminus of the Vps10p cytosolic domain resulted in secretion of CPY. The level of CPY secretion was significant in strains containing deletions encompassing amino acids 75–85 (Fig. 8, lanes *7–12*). The role for this region in CPY sorting was confirmed with an internal deletion (Δ77–85), which resulted in 56% CPY missorting (lanes *15* and *16*). This region contains the sequence $Y_{77}SSLI_{81}$, which is conserved between Vps10p and Vth2p, and includes both an aromatic residue and a dileucine motif, both of which have been shown to be involved in the trafficking of the mammalian M6PR (Lobel, 1989; Canfield, 1991; Johnson and Kornfeld, 1992). In addition, a second aromatic residue in this region, F_{69}, is conserved between Vps10p and Vth2p. We constructed various amino acid substitutions in the Vps10p cytosolic domain and examined their effect on CPY secretion (Fig. 8). The substitution $Y_{77}A$ resulted in 36% CPY secretion, whereas the $F_{69}A$ substitution failed to result in a CPY missorting phenotype (data not shown). The double substitution $L_{80}A$ $I_{81}A$ resulted in 12% secretion of CPY raising the possibility that, as is the case for the M6PR, Vps10p trafficking signals contain both an aromatic residue component as well as a dileucine motif. To address this issue the single substitution mutations $L_{80}A$ and $I_{81}A$ were constructed. Yeast cells expressing Vps10p-$I_{81}A$ (AACY26) correctly sort CPY, whereas yeast expressing Vps10p-$L_{80}A$ (AACY25) failed to sort CPY efficiently, indicating the importance of L_{80} for Vps10p function and eliminating the possibility that $L_{80}I_{81}$ constituted a dileucine motif.

These results indicate that the sequence $Y_{77}SSL_{80}$ is an important component of the Vps10p recycling signal, however, mutations within this signal result in the missorting of 35–45% of CPY, which suggests that a second signal may exist that contributes to the trafficking of Vps10p. Cells expressing Vps10p-100* secreted 19% of the CPY synthesized, inferring that a potential second signal may exist in the region of amino acids 100–164. To test whether the conserved F_{106} residue (see Fig. 7 *A*) is this second signal, the mutation $F_{106}A$ was generated in *VPS10*. Yeast cells expressing the mutant receptor (Vps10p-$F_{106}A$) missorted 20% of CPY (Fig. 8, lanes *21* and *22*). Since the level of secretion of cells expressing Vps10p-100* (19%) or Vps10p-$F_{106}A$ (20%) was very similar, it is likely that F_{106} represents the important element for receptor recy-

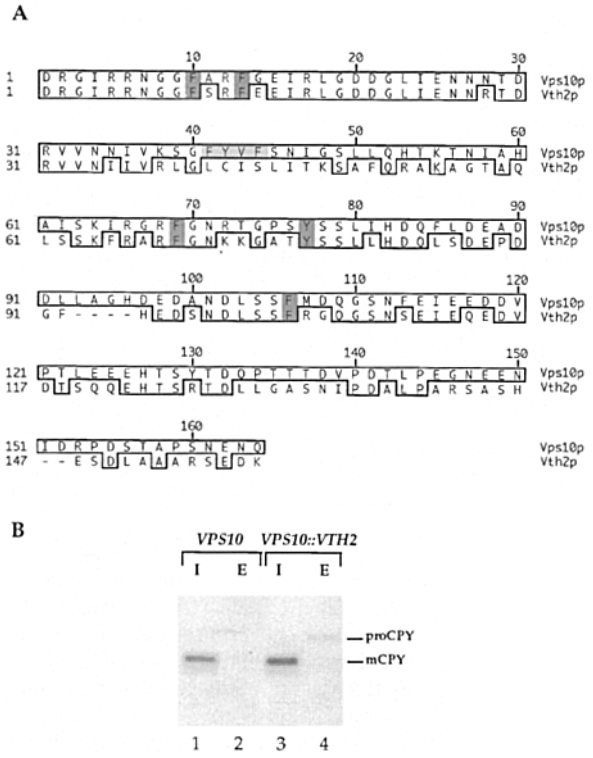


Figure 7. Comparison and functional substitution of the Vps10p and Vth2p cytosolic domains. (*A*) Schematic representation of the amino acid residues of the Vps10p and Vth2p cytosolic domains. The two domains share 60% amino acid identity. Amino acid residue numbering begins with the first amino acid following the transmembrane domain (1) to the end of the cytosolic domain (164) of Vps10p. Boxed residues indicate residues identical to those of the Vps10p cytosolic domain. Dark shaded residues highlight aromatic amino acids conserved between Vps10p and Vth2p cytosolic domains while the lightly shaded region shows the $FYVF_{44}$ cluster of aromatic residues present only in Vps10p. (*B*) Strains RPY10 (*VPS10*, lanes *1–2*) and AACY50 (*VPS10::VTH2*, lanes *3–4*) were radiolabeled for 10 min and chased for 30 min before harvest. CPY was immunoprecipitated and analyzed by SDS-PAGE and fluorography as described in Fig. 1.

cling in the last 64 amino acids of the Vps10p cytosolic domain. A double mutation was constructed that contained both the $Y_{77}A$ and $F_{106}A$ substitutions and this resulted in ~50% CPY secretion (Fig 8, lanes *23* and *24*).

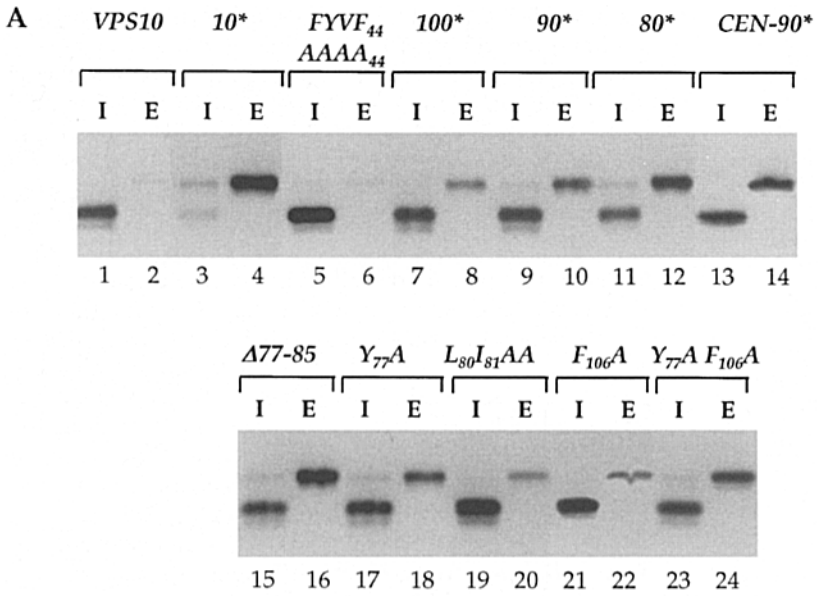
The Y_{77}, F_{106} signal plays a major role in the membrane trafficking of Vps10p yet other residues are likely to contribute. The central role that aromatic residues can play in sorting led us to investigate the role of a cluster of aromatic amino acids ($FYVF_{44}$) within the cytosolic domain of Vps10p. Although not shared with Vth2p (Fig. 7 *A*), FYVF is similar to signals involved in the trafficking of the yeast Golgi proteins DPAP A and Kex2p (Nothwehr and Stevens, 1994). To test whether this motif is involved in the recycling of Vps10p, we mutagenized the $FYVF_{44}$ sequence to $AAAA_{44}$. Vps10p-$AAAA_{44}$ was found to sort CPY to the vacuole with the same efficiency as wild-type Vps10p (Fig. 8, lanes *5* and *6*). The sequence $FYVF_{44}$ was not conserved in the Vth2p cytosolic domain (Fig. 7 *A*), already shown to functionally substitute for the Vps10p domain, further supporting the conclusion that this motif is not important for Vps10p function.

During preparation of this manuscript it was reported that a deletion of the $FYVF_{44}$ motif in *VPS10* (when expressed on a plasmid) resulted in a significant missorting of CPY (Cereghino et al., 1995), which was in conflict with our findings. To address this discrepancy we created the $\Delta(FYVF_{44})$ mutant allele, and, along with the wild-type and $FYVF_{44}$ to AAAA alleles of *VPS10*, expressed it on a low copy plasmid. As described above, the plasmid borne *VPS10* does not fully complement a *vps10Δ* mutation, yet the plasmid borne wild-type as well as $AAAA_{44}$ *VPS10* alleles permitted *vps10Δ* cells to sort CPY to a similar degree (<25% secreted, Fig. 9, lanes *1–4*). However, over 60% of the CPY was secreted by the strain expressing the $\Delta(FYVF_{44})$ allele (Fig. 9, lanes *5–6*). Given these results it seems likely that the secretion defect ascribed to the $\Delta(FYVF_{44})$ mutation by Cereghino et al. (1995) was presumably due to a structural alteration of the cytosolic domain rather than to the absence of the $FYVF_{44}$ motif.

The degree of CPY secretion by the mutant forms of Vps10p served as a good indicator of the efficiency with which the receptor recycled back to the Golgi. However, a more direct method of assessing recycling was to measure the half-time of Vps10p proteolytic cleavage by vacuolar proteases. A failure of Vps10p to recycle to the Golgi results in its delivery to the vacuole where it is cleaved in a *PEP4*-dependent manner. Thus, the proteolytic cleavage half-times of the various mutant forms of Vps10p should reflect the extent to which the various proteins are recycled. Wild-type Vps10p was very stable during a 3-h chase, whereas Vps10p-10* had a cleavage half-time of ~25 min (Fig. 5 *B*; Table II; Cereghino et al., 1995). As expected, forms of Vps10p carrying more subtle mutations in the cytosolic domain also underwent proteolytic cleavage reflecting transport to the vacuole. In general, there was a strong correlation between the degree of CPY missorting and the rate of Vps10p proteolytic cleavage for the Vps10p mutant proteins analyzed (Table II). Forms of Vps10p that sorted CPY inefficiently (e.g., Vps10p-75* or Δ77-85) exhibited short half-times of proteolytic cleavage. While our assay for Vps10p recycling remains indirect, these data strongly suggest that the cytosolic domain mutations interfered with the recycling of Vps10p.

Discussion

The data presented in this paper, when taken together with the work of Emr and colleagues (Marcusson et al., 1994), demonstrate that *VPS10* encodes the yeast sorting receptor for vacuolar carboxypeptidase Y. The stoichiometry of proCPY binding is such that Vps10p must recycle numerous times between the Golgi and prevacuolar compartments to sort the excess CPY. This trafficking route requires the cytosolic domain of Vps10p, since its removal resulted in a receptor protein that was mislocalized and failed to sort CPY. A tyrosine-based signal within the cytosolic domain of Vps10p, similar to that of the M6PR, plays an essential role in retrieval of Vps10p to the Golgi. A homologue of the receptor, Vth2p, was also found capable of sorting CPY.



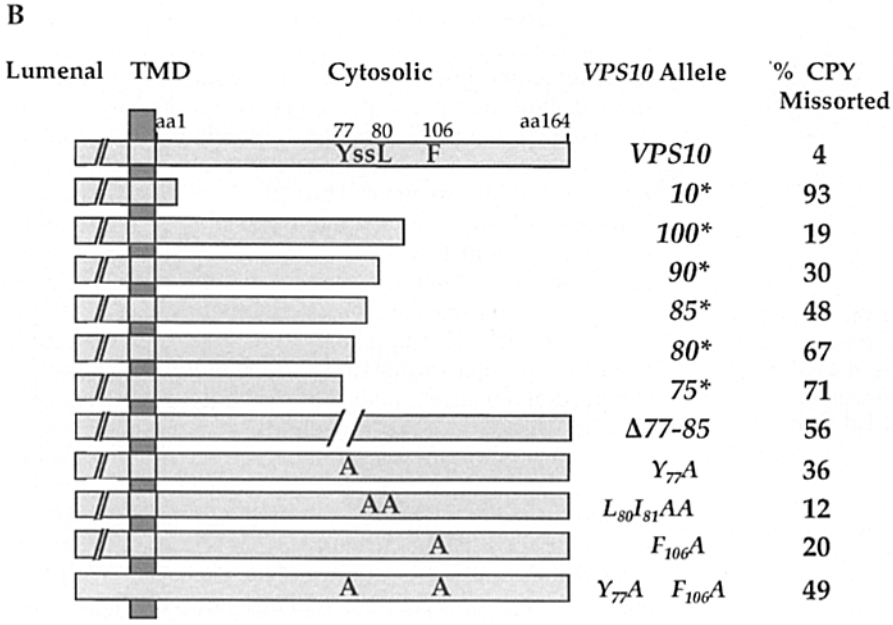


Figure 8. Mutations in the Vps10p cytosolic domain affect CPY sorting. (*A*) Strains bearing various truncations of, or substitutions in, the cytosolic domain of Vps10p were radiolabeled for 10 min and chased for 30 min before immunoprecipitation of CPY from intracellular (*I*) and extracellular (*E*) fractions, followed by analysis by SDS-PAGE and fluorography. (*B*) Schematic representation of the Vps10p lumenal, transmembrane (TMD), and cytosolic domains indicating the extent of truncations and/or amino acid substitutions. CPY immunoprecipitated in the above experiments (Fig. 8 *A*) was quantified and used to calculate the percent of CPY missorted in the various mutants. Amino acid numbering begins with the first amino acid of the cytosolic domain. Amino acids 77-81 ($YSSLI_{81}$) and 106 (F) of the cytosolic domain are highlighted. The * symbol represents the introduction of a stop codon in place of the listed Vps10p cytosolic domain codon.

Vps10p Sorts the Soluble Vacuolar Hydrolases CPY and PrA

Consistent with earlier work, *vps10Δ* cells were found to secrete greater than 90% of newly synthesized CPY (Marcusson et al., 1994). However, in contrast to previous results (Marcusson et al., 1994), *vps10Δ* cells also secreted ~50–60% of PrA, another soluble vacuolar hydrolase, implying that Vps10p sorts both CPY and PrA to the vacuole. Our results demonstrating that *vps10Δ* cells secrete >50% of PrA are in close agreement with the original paper by Emr and colleagues describing the isolation of *vps10* alleles where such mutants were found to secrete a high percentage of CPY (~85%) and approximately half of the PrA (~45%) (Robinson et al., 1988). Vps10p is likely to be directly involved in the sorting of PrA as overexpression of PrA led to the secretion of CPY, presumably due to saturation of Vps10p, whereas overproduction of PrB had no effect. In addition, the missorting of PrA in *vps10Δ* cells was partially suppressed by the overproduction of Vth2p which was also able to sort CPY, which further demonstrates that these receptors are capable of binding/sorting several ligands. Consistent with Vps10p sorting both hydrolases is the proposal that CPY and PrA share similar vacuolar sorting signals within their propeptides (McIntyre et al., 1994).

A second receptor, capable of sorting PrA but not CPY, may be responsible for correctly sorting the remaining 50% of PrA to the vacuole in *vps10Δ* cells. In fact, we have identified two new yeast genes that exhibit extensive sequence identity to Vps10p (Cooper, A.A., and T.H. Stevens, unpublished data). Neither of these proteins is required for the sorting of CPY or PrA, however, overexpression of Vth2p can suppress the CPY and PrA sorting defects associated with *vps10Δ* cells. In addition, the Vth2p cytosolic domain can substitute functionally for the cytosolic domain of Vps10p, suggesting that Vth2p cycles between the

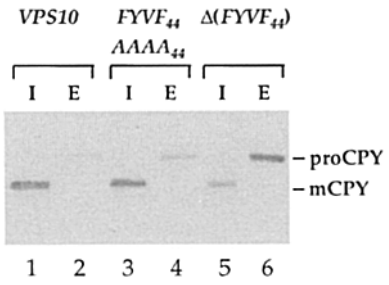


Figure 9. The FYVF cluster of aromatic residues does not contribute to the Vps10p cytosolic signal. AACY9 (*vps10Δ*) was transformed with alleles of *VPS10* on a centromere plasmid: wild-type *VPS10* (lanes *1* and *2*), *vps10-FYVF-AAAA* (lanes *3* and *4*) and *vps10-Δ(FYVF)* (lanes *5* and *6*). These transformants were radiolabeled for 10 min and chased for 30 min before immunoprecipitation of CPY from intracellular (*I*) and extracellular (*E*) fractions, followed by analysis by SDS-PAGE and fluorography.

Golgi and prevacuolar compartments and sorts additional vacuolar hydrolases.

VPS10 had previously been proposed to encode a membrane protein directly involved in the binding and sorting of CPY, since Vps10p could be cross-linked to proCPY but not to a missorted mutant form of CPY (Marcusson et al., 1994). Our in vitro equilibrium binding analysis complements the cross-linking data and has confirmed this model and further shown that the stoichiometry of binding between proCPY and Vps10p is 1:1.

In the future, a more detailed analysis of proCPY binding to Vps10p should allow us to determine the proCPY binding constant as well as the conditions that affect binding. Ionic conditions that modulate binding in vitro might reflect important differences between the lumen of the yeast late-Golgi and prevacuolar compartments that are responsible for the cycle of binding/dissociation in vivo. A pH difference between the mammalian TGN and acidified endosomal compartment is responsible for the dissociation of lysosomal hydrolases from the M6PR (Kornfeld, 1992). However, in yeast, the role of acidification in CPY sorting is far from clear. Disruption of *VMA* genes encoding subunits of the vacuolar proton translocating H^+-ATPase abolishes the relevant ATPase activity and results in a neutral pH vacuole. Several *vmaΔ* mutant strains secrete a minor portion of CPY (20–25%; Yamashiro et al., 1990), whereas other *vma* mutant strains either secrete higher levels of CPY (25–50%) or exhibit a delay in CPY maturation (Klionsky et al., 1992; Morano and Klionsky, 1994).

Table II. Mutations in the Cytosolic Domain of Vps10p Reduce Its Stability

	CPY Secretion‡	Vps10p Stability§
	%	*min*
Vps10p	4	>>240
Vps10p-10*	93	~25
Vps10p-80*	67	~75
Vps10p-Δ(77-85)	56	~90
Vps10p-$Y_{77}AF_{106}A$	49	~100
Vps10p-$Y_{77}A$	36	~110

‡Percentages of CPY secretion are taken from Fig. 8.
§Vps10p stability represents the half-time of proteolytic cleavage of Vps10p as shown in Fig. 5 *B*.

Vps10p Recycles between the Late Golgi and the Prevacuolar Compartment

Biochemical analysis has indicated that sorting of proCPY occurs in the late-Golgi complex in the Kex2p-containing, or potentially later, Golgi compartment (Graham et al., 1991; Vida et al., 1993; Stack et al., 1995). Subcellular fractionation has assigned Vps10p to a membrane fraction containing Golgi and possibly prevacuolar/endosomal compartments (Marcusson et al., 1994; Cereghino et al., 1995; Cooper, A.A, and T.H. Stevens, unpublished data). Our immunofluorescence studies localized a significant fraction of Vps10p to the Golgi complex consistent with its proposed site of binding proCPY. The steady-state distribution of Vps10p in the Golgi combined with the >20:1 ratio of newly synthesized ligand:receptor supports the idea that Vps10p must recycle from the prevacuolar compartment to the Golgi apparatus to sort the excess CPY. Furthermore, wild-type Vps10p is a very stable protein suggesting that its normal trafficking route does not expose it to vacuolar hydrolases. This suggests that CPY dissociates from the receptor in a prevacuolar compartment, from which CPY is then delivered to the vacuole and Vps10p is recycled back to the late-Golgi compartment for another

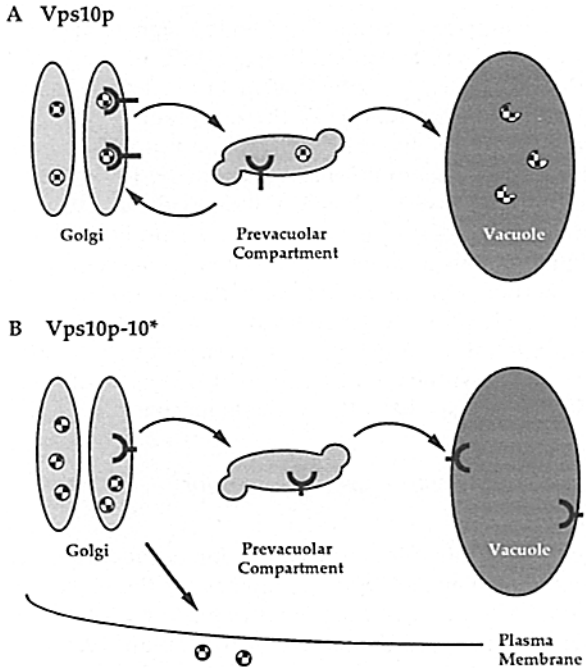


Figure 10. Model of Vps10p membrane trafficking. (*A*) proCPY (*checked circle*) binds Vps10p (*trident shaped receptor*) in the late-Golgi and is sorted to the prevacuolar compartment whereupon it dissociates from Vps10p. proCPY is delivered to the vacuole where it is activated (checked circle with sector missing). Vps10p is recycled back to the Golgi through sorting signals in its cytosolic domain to sort more proCPY. (*B*) Vps10-10* remains capable of binding proCPY, but lacking a sorting signal in its cytosolic domain (trident shaped receptor with shorter shaft), it does not recycle and instead is delivered to the vacuole. The depletion of Vps10p from the late-Golgi results in secretion of proCPY.

round of sorting (Fig. 10 *A*). This is analogous to the trafficking pathway of the M6PR, which cycles between the *trans*-Golgi and the endosome. Recent evidence indicates that a prevacuolar compartment exists in yeast and that Vps10p traffics through this organelle (Piper et al., 1995).

Deletion of the Vps10p cytosolic domain (Vps10p-10*) resulted in delivery of the protein to the vacuole and a loss of CPY sorting (Fig. 10 *B*). Overexpression of Vps10p-10* did not restore CPY sorting due to increased recycling of Vps10p-10*, but rather the enhanced sorting efficiency was likely due to an increased flux of ligand-bound receptor traveling from the Golgi to the vacuole.

The issue of whether Vps10p departs the Golgi constitutively or only in a ligand-bound state is an open question at this stage. Vps10p could constitutively recycle as do proteins such as the transferrin receptor, α–2-macroglobulin receptor, asialoglycoprotein receptor, and M6PR (Pfeffer, 1987; Jin et al., 1989). Alternatively, Vps10p could remain in the sorting compartment until proCPY binds, whereupon a signal is transduced to mediate departure of the complex from the Golgi compartment (Herman et al., 1992; Stack et al., 1995). In this "Sortosome" model of Emr and colleagues, the signal for Golgi exit would be transduced through the cytosolic domain. However, Vps10p-10* (which lacks the cytosolic domain) is capable of binding proCPY and does not require the cytosolic domain for exiting the Golgi and delivery to the vacuole.

The enzymes Kex1p, Kex2p, and DPAP A reside in a late-Golgi compartment due to retention signals in their cytosolic domains (Cooper and Bussey, 1992; Wilcox et al., 1992; Roberts et al., 1992; Nothwehr et al., 1993). It has been proposed that retention of these proteins within the Golgi apparatus involves retrieval from a post-Golgi prevacuolar compartment (Wilsbach and Payne, 1993; Nothwehr and Stevens, 1994). However, little evidence exists to discriminate between a retrieval mechanism and a static retention mechanism where proteins have a long residence time in the Golgi and are effectively excluded from vesicles bound for a prevacuolar compartment (Nothwehr and Stevens, 1994). Since Vps10p must recycle rapidly to sort all of the newly synthesized CPY, Vps10p may be localized to the Golgi exclusively by the retrieval mechanism.

Aromatic-based Signals Are Required for Vps10p Recycling

Mutagenesis of the Vps10p cytosolic domain identified a tyrosine-based signal, $Y_{77}SSL_{80}$, required for the recycling of Vps10p. Alteration of this signal reduced CPY sorting efficiency and resulted in delivery of the mutant receptor to the vacuole. This signal is very similar to the tyrosine-based internalization signals that govern the endocytosis of many mammalian recycling receptors by clathrin-coated pits (Trowbridge et al., 1993). These signals encompass a tetrapeptide sequence containing an aromatic residue in the first position and a large hydrophobic residue at the fourth position. Such a signal has also been implicated in the direct intracellular sorting of the M6PR from the TGN to prelysosome indicating that the signal present in Vps10p may be functionally very similiar to that of the M6PR. The $YSSL_{80}$ signal of Vps10p is also very similar to the signal (SXYQRL) responsible for localizing the membrane protein TGN38 to the mammalian TGN (Wong and Hong, 1993), the functional equivalent of the yeast late-Golgi compartment (Wilsbach and Payne, 1993).

The F_{106} residue within the Vps10p cytosolic domain is also conserved in the cytosolic domain of Vps10p homologue, Vth2p, and contributes a second retrieval signal. The presence of a second sorting signal (YTRF) has been found to enhance the internalization rate of the transferrin receptor (Collawn et al., 1993). Thus, Vps10p may contain two separate retrieval signals to either enhance its recycling rate or to improve the efficiency of its recycling. Whereas other regions of the *VPS10* cytosolic domain have been implicated in targeting ($FYVF_{44}$; Cereghino et al., 1995) they are likely to play only a structural role.

The sorting machinery responsible for recognizing the $YSSL_{80}$ sorting signal within the Vps10p cytosolic domain may include clathrin, since the comparable signal on the M6PR is known to bind clathrin/adaptins (Pearse and Robinson, 1990; Kornfeld, 1992). In support of this idea yeast mutants with temperature-sensitive alleles of the clathrin heavy chain gene ($chc1^{ts}$) secrete a significant amount of newly synthesized CPY and PrA after a short time at the nonpermissive temperature (Seeger and Payne, 1992*a*). A second consequence of incubating $chc1^{ts}$ cells at the restrictive temperature is the mislocalization of the Golgi membrane proteins, Kex2p and DPAP A (Seeger and Payne, 1992*b*). These two observations are consistent with a role for clathrin in the sorting of CPY as loss of clathrin function may also mislocalize the late-Golgi protein, Vps10p, which would result in the missorting of CPY.

The machinery responsible for recycling of Vps10p appears to be saturable as overproduction of wild-type Vps10p resulted in delivery of the protein to the vacuole (Cereghino et al., 1995; Cooper, A.A., and T.H. Stevens, unpublished data). In addition, this machinery might be shared with the cytoplasmic domains of other late-Golgi proteins, since overproduction of Kex2p but not Kex2pΔC (Kex2p lacking the cytosolic domain) resulted in mislocalization of Vps10p (Cooper, A.A., and T.H. Stevens, unpublished data). These latter observations suggest a strategy for the identification of proteins responsible for binding the Vps10p sorting signals and effecting receptor recycling.

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