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 cytosolic domain of Vps10p 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 (YSSLs0) 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 vpsl10A 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) 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 prevacuolar/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). 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 PEPI/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 luminal 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 luminal 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 pre-vacuolar 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 (% yeast extract, % peptone, % dextrose; YEPD) or standard minimal medium (SD) with appropriate supplements as described by Sherman et al. (1986). Strain TSY108 was constructed by transforming SF388-SA (MATa 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 Clal (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 (Robert 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 (Gathersburg, 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 Amer sham 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 (IgG sorb) were obtained from The Enzyme Center (Malden, MA). 35S-Express label was from New England Nuclear (Boston, MA). Oxalylcine was from Enzymogenetics (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 |
|--------|----------|---------------|
| SF388-SA | MATa ura3-52, leu2-3,112 his4-519 ade6 pep4-3 | |
| RPY10 | MATa ura3-52, leu2-3,112 his4-519 ade6 | SF388-SA |
| AACY10 | vps10Δ::URA3 pepd-4 | SF388-SA |
| AACY29 | vps10-10* pepd-4 | SF388-SA |
| AACY9 | vps10Δ::URA3 | RPY10 |
| AACY11 | vps10-60* | RPY10 |
| AACY12 | vps10-100* | RPY10 |
| AACY13 | vps10-85* | RPY10 |
| AACY15 | vps10-180* | RPY10 |
| AACY16 | vps10-10* | RPY10 |
| AACY17 | vps10-FFV4AAA | RPY10 |
| AACY19 | vps10-90* | RPY10 |
| AACY20 | vps10-75* | RPY10 |
| AACY21 | vps10-160A l60A | RPY10 |
| AACY22 | vps10-Δ77-85 | RPY10 |
| AACY23 | vps10-160A l60A | RPY10 |
| AACY24 | vps10-Δ155 | RPY10 |
| AACY25 | vps10-160A | RPY10 |
| AACY26 | vps10-160A l60A | RPY10 |
| AACY27 | vps10-160A l60A | RPY10 |
| AACY28 | pho8Δ::LEU2 | RPY10 |
| AACY40 | vps10-γ9, F69A | RPY10 |
| AACY50 | VPS10::VTTH2 | RPY10 |
| SEY6210 | MATa ura3-52, leu2-3,112 his3-Δ200 prp1-901 lys2-801 suc2-9 | |
| AACY30 | vps10Δ::URA3 | SEY6210 |

1Rothman et al. (1989).
2Robinson et al. (1988).

Strains AACY5, AACY30, 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 Smal site of YEp352 (Hill et al., 1986) to produce pKE50. Flanking Sall and SacI sites in pKE50 permitted the 6.1-kb fragment containing VPS10 to be inserted into pRS315 (Sikorski and Hieter, 1989) cut with Sall and SacI to generate plasmid pAAC220. The vps10Δ-URA3 allele used to disrupt the VPS10 locus was constructed as follows: pAAC214 contains the 6.1-kb Sall–SacI fragment of VPS10 inserted into pBluescript II KS+ (Stratagene, La Jolla, CA) cut with Sall and SacI. Site-directed mutagenesis was performed using the bacterial strain CJ236 and the helper plage M13K07 according to the method of Kunzel et al. (1987) to introduce a BglII site immediately downstream of the VPS10 initiation codon thereby creating plasmid pAAC215. This plasmid was cut with Stul 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 pE485. The resulting plasmid, pAAC220, was cleaved with SacI and XhoI before transformation into RPY10 and SP388-9D to create yeast strains AACY9 (vps10Δ-URA3) and AACY10 (vps10Δ-URA3 pep4-3), respectively. A CPA overlay blot (Roberts et al., 1991; Piper et al., 1994) was used to identify the 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 KS+ cut with the same enzymes to generate plasmid pAAC219. Single stranded DNA derived from pAAC219 was used in oligonucleotide directed mutagenesis to create the substitution mutation FYIVF44 to AAAA44. 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–Stul fragment of VPS10 that encodes the cytoplasmic domain was amplified from wild-type VPS10 DNA with oligonucleotides containing the desired mutation FYIVF44 or AAAA44 for two-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 Stul before insertion into pAAC220 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).

The remaining mutations introduced into the Vps10p transmembrane domain of VPS10 were accomplished by PCR amplification. The 4.8-kb PstI–Stul fragment containing the cytoplasmic domain of Vps10p was inserted into the SmaI–XbaI fragment of PCR315 (Sikorski and Hieter, 1989) cut with Stul 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 pE485. The resulting plasmid, pAAC220, was cleaved with SacI and XhoI before transformation into RPY10 and SP388-9D to create yeast strains AACY9 (vps10Δ-URA3) and AACY10 (vps10Δ-URA3 pep4-3), respectively. A CPA overlay blot (Roberts et al., 1991; Piper et al., 1994) was used to identify the 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.

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 (pho8Δ4) 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 a T-MAX 400 film.

Immunoaffinity micrographs and autoradiograms were digitized with a Scanalyzer (Reli 4816; Mileipas, CA). Images within the same experiment were processed with Adobe Photoshop™ using identical settings and printed on a Tectors 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 pAAC221. After signal sequence cleavage, the soluble form of Vps10p encoded by pAAC231 is predicted to have the following amino-terminal sequence REFPSHHHHHHHT 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 KPO4, pH 8.0, 50 mM NaCl to raise the supernatant pH to approximately pH 7.5. The supernatant was then loaded onto a Ni2+-NTA-Agarose (Qiagen, Chatsworth, CA) column and washed with 50 mM KPO4, pH 8.0, 250 mM NaCl before elution with 50 mM KPO4, pH 8.0, 50 mM NaCl. 200 mM imidazole. The eluted material was dialyzed against 50 mM NaCl.
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 Sorrensen, 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 SLMAMINCO spectrophotometer, 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 1 and 2), whereas the parent strain SF838-5A correctly sorted CPY (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 (Marcussen 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 (Marcussen 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

![Figure 1](image-url)

**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.

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 luminal 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 2](image-url)

**Figure 2.** Binding of proCPY by the luminal 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 luminal domain of Vps10p (lane 1) and proCPY (lane 2) were analyzed by SDS-PAGE and staining with Coomassie brilliant 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-c-CPY 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 3SS-Express label.
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Localization and CPY Sorting

The yeast late-Golgi membrane proteins Kexlp, Kex2p, and DPAP A achieve their Golgi localization through interaction between Vpsl0p and proCPY:Vpsl0p binding stoichiometry would also be 1:1. The disruption of both vpslOA and vpslO 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). 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 VpslOp Cytosolic Domain Is Essential for VpslOp Localization and CPY Sorting

The yeast late-Golgi membrane proteins Kex1p, Kex2p, and DPAP A achieve their Golgi localization through interactions with the cytosolic domain of Vpsl0p. Disruption of the vps10Δ strain might sort 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. A similar strategy was employed to determine if wild-type levels Vth2p contribute to the sorting efficiency of CPY. 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.
Vps10p 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.

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 I–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.
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.

To identify the recycling signal of Vps10p, a mutagenic analysis of the Vps10p cytosolic domain was performed. It was determined whether 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
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 Y77SSL81, 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, F69, 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 Y77A resulted in 36% CPY secretion, whereas the F69A substitution failed to result in a CPY missorting phenotype (data not shown). The double substitution L80A I81A 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 L80A and I81A were constructed. Yeast cells expressing Vps10p-80A (AACY26) correctly sort CPY, whereas yeast expressing Vps10p-L80A (AACY25) failed to sort CPY efficiently, indicating the importance of L80 for Vps10p function and eliminating the possibility that L80I81 constituted a dileucine motif.

These results indicate that the sequence Y77SSL80 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 F106 residue (see Fig. 7A) is this second signal, the mutation F106A was generated in VPS10. Yeast cells expressing the mutant receptor (Vps10p-F106A) missorted 20% of CPY (Fig. 8, lanes 21 and 22). Since the level of secretion of cells expressing Vps10p-100* (19%) or Vps10p-F106A (20%) was very similar, it is likely that F106 represents the important element for receptor recy-
cloning in the last 64 amino acids of the Vps10p cytosolic domain. A double mutation was constructed that contained both the Y77A and F106A substitutions and this resulted in ~50% CPY secretion (Fig. 8, lanes 23 and 24).

The Y77, F106 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 (FYVF44) within the cytosolic domain of Vps10p. Although not shared with Vth2p (Fig. 7A), 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 FYVF44 sequence to AAAA44. Vps10p-AAAA44 was found to sort CPY to the vacuole with the same efficiency as wild-type Vps10p (Fig. 8, lanes 5 and 6). The sequence FYVF44 was not conserved in the Vth2p cytosolic domain (Fig. 7A), 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 FYVF44 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 Δ(FYVF44) mutant allele, and, along with the wild-type and FYVF44 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 AAAA44 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 Δ(FYVF44) allele (Fig. 9, lanes 5–6). Given these results it seems likely that the secretion defect ascribed to the Δ(FYVF44) mutation by Cereghino et al. (1995) was presumably due to a structural alteration of the cytosolic domain rather than to the absence of the FYVF44 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. 9B; 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 (Marcussen 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.

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**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 FYVF44 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.
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).

**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/endoosomal 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 suggests 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 cycle.

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

| Mutations      | CPY Secretion | Vps10p Stability |
|----------------|---------------|------------------|
| Vps10p         | 4%            | >>240            |
| Vps10p-10*     | 93%           | ~25              |
| Vps10p-80*     | 67%           | ~75              |
| Vps10p-Δ(77-85)| 56%           | ~90              |
| Vps10p-Y77AF106A| 49%          | ~100             |
| Vps10p-Y77A    | 36%           | ~110             |

1Percentages of CPY secretion are taken from Fig. 8.
2Vps10p stability represents the half-time of proteolytic cleavage of Vps10p as shown in Fig. 5 B.

**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) Vps10p-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.
Aromatic-based Signals Are Required for Vps10p Recycling

Mutagenesis of the Vps10p cytosolic domain identified a tyrosine-based signal, Y_{SSL80}, 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 signal, Y_{SSL80}, required for the recycling of CPY. Vps10p may be localized to the Golgi exclusively by the retrieval mechanism.

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 and Stevens, 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_{SSL80}, 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 also plays an important role in the direct intracellular sorting of the M6PR from the TGN to prelysosome indicating that the signal present in Vps10p may be functionally very similar to that of the M6PR. The Y_{SSL80} 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_{1066} residue within the Vps10p cytosolic domain is also conserved in the cytosolic domain of Vps10p homologues, Vth2p, and contributes a second retrieval signal. The presence of a second sorting signal (Y_{TRF}) 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 Y_{SSL80} 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 (chcl) secrete a significant amount of newly synthesized CPY and PrA after a short time at the nonpermissive temperature (Seeger and Payne, 1992a). A second consequence of incubating chcl cells at the restrictive temperature is the mislocalization of the Golgi membrane proteins, Kex2p and DPAP A (Seeger and Payne, 1992b). 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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