INTRODUCTION

The secretory pathway mediates the proper delivery and sorting of proteins to a variety of subcellular compartments. The vacuole of the budding yeast *Saccharomyces cerevisiae* is functionally equivalent to the mammalian lysosome, and both are acidic compartments involved primarily in the degradation of macromolecules (Klionsky et al., 1990; Kornfeld & Mellman, 1989). The delivery of proteins to the vacuolar compartment is mediated by the secretory pathway and is one of the best-characterized examples of an intracellular protein-sorting process in *S. cerevisiae*. Genetic selections in *S. cerevisiae* have resulted in the isolation of a large number of mutants defective in vacuolar protein sorting (Bankaitis et al., 1986; Robinson et al., 1988; Rothman et al., 1989; Rothman & Stevens 1986). These *vps* (vacuolar protein sorting) mutants missort and secrete the precursors of soluble vacuolar hydrolases, such as carboxypeptidase Y (CPY) and proteinase A (PrA).

In mammalian cells, soluble lysosomal proteins are inserted into the endoplasmic reticulum (ER) and then transported through the Golgi apparatus where they receive a mannose 6-phosphate moiety that acts as a recognition signal for the mannose 6-phosphate receptor. The mannose 6-phosphate–lysosomal protein complexes are sorted into transport vesicles that fuse with the endosome. The low pH of the endosome triggers release of lysosomal proteins from the receptor and these proteins are eventually sorted to the lysosome (Kornfeld, 1992). The mechanism by which soluble proteins, such as CPY, reach the vacuole in *S. cerevisiae* appears to be very similar to the mechanism of lysosomal protein sorting in mammalian cells. CPY is cotranslationally translocated into the ER, where its core is glycosylated to generate the p1 precursor form of CPY. It next traverses the Golgi complex, where its oligosaccharides are elongated to create p2CPY. In the Golgi apparatus, a CPY sorting signal is recognized and a receptor–ligand complex is formed. The vacuolar sorting signal for CPY is located in the polypeptide (Johnson et al., 1987; Valls et al., 1987, 1990), and the pro-peptide sequence is the targeting signal for receptor recognition in the late Golgi (van Voorst et al., 1996; Valls et al., 1990). The receptor has been identified as being encoded by...

Abbreviations: CFP, cyan fluorescent protein; CPY, carboxypeptidase Y; ER, endoplasmic reticulum; GFP, green fluorescent protein; PrA, proteinase A; PrB, proteinase B; PVC, prevacuolar compartment; TGN, trans-Golgi network; YFP, yellow fluorescent protein.
the VPS gene, VPS10/PEP1. VPS10 encodes a type I transmembrane receptor protein responsible for recognition and targeting of CPY to the vacuole (Marcussen et al., 1994). Precursor CPY can bind to Vps10p in the late Golgi compartment (Marcussen et al., 1994; Cooper & Stevens 1996). Receptor–ligand complexes are delivered to an intermediate endosomal compartment, where CPY dissociates from Vps10p. Vps10p cycles back to the Golgi for additional rounds of sorting while CPY continues on to the vacuole (Takegawa et al., 2003b). This result suggests that Vps10p is required for targeting Cpy1p to the vacuole (Tabuchi et al., 1997a) and Takegawa et al. (1995). The vps5Δ (h0 leu1 ura4–D18 ade6–M210 vps5::ura4+), vps17A (h0 leu1 ura4–D18 ade6–M210 his2 vps17::ura4+), vps53Δ (h0 leu1 ura4–D18 ade6–M210 vps53::ura4+) mutants were obtained from Dr Y. Fukushima (Tokyo University). Standard rich medium containing YES (5 g l−1 yeast extract, and 30 g l−1 glucose) orYPD (10 g l−1 yeast extract, 5 g l−1 peptone and 20 g l−1 glucose), and synthetic minimal medium (MM) were used for growing Sch. pombe cells as described by Moreno et al. (1991). Sch. pombe was transformed by the lithium acetate method or electroporation (Okazaki et al., 1990; Suga et al., 2000). Standard genetic methods have been described previously (Alfa et al., 1993).

Plasmid constructs. Conventional recombinant DNA methods were used in the construction and propagation of all plasmids (Sambrook et al., 1989). The Sch. pombe shuttle vector pAL-KS+ was kindly provided by C. Shimoda (Osaka City University, Japan), pST1 was constructed by inserting the subcloned 5.6 kb genomic DNA fragment of vps10+ into pAL-KS+. Oligonucleotide-directed mutagenesis of the vps10+ gene was performed in a dut ung E. coli mutant as described by Kunkel et al. (1991). The vps10+–containing plasmid was introduced into the dut ung E. coli strain CJ236, and single-stranded plasmid DNA was isolated and mutagenized as described in the Takara Mutan K kit manual. Primer 5′-CGATATGC-CTGATTACTAGTGTATTTGAC-3′ was used to replace leucine1408 with a stop codon and introduce the Spel site (underlined), resulting in pST1-A59.

To tag Sch. pombe Vps10p (SpVps10p) with GFP, the vps10+ ORF was amplified by PCR and subcloned into pTN197, a derivative of the thiamine-repressible expression vector pREP41 (Nakamura et al., 2001), resulting in pTN197-vps10. To create SpVps10p–YFP, the vps10+ ORF, under control of the attenuated nmt1 promoter, was amplified from pTN197-vps10 and subcloned into the Apal and Smal sites of pAL-KS+. Then YFP from pYFP (BD Biosciences Clontech) was inserted into the Smal and NotI sites, resulting in pAL-nmt1–vps10–YFP. To tag Gms1p with CFP, a 13-kb Smal–BanIII fragment encoding gene gms1+ and its promoter was subcloned into pAU-KS+. A BanIII–NotI fragment encoding CFP from pECFP (BD Biosciences Clontech) was then inserted, resulting in pAU-gms1-CFP.

The remaining mutations introduced into the Spvps10p cytoplasmic domain were accomplished by PCR. pST1 and pTN197-vps10 were amplified by KOD-Plus-DNA polymerase (TOYOBO) and self-ligated. The following primers were used to replace phenylalanine (F) 1419 with alanine (A): (F1419A): 5′-TGCTTCTGATCAATTCATGGATCTTTTCCGC-3′ and 5′-GCTCCTCTTGATCTAGATGCGCTTACC-3′; F1422 with A (F1422A): 5′-CAATATGCCTTTAAGCTTTACAAAGC-3′, F1422 with A (F1422A): 5′-CAATATGCCTTTAAGCTTTACAAAGC-3′, F1422 and F1426 with A (F1422A, 1426A): 5′-CAATATGCCTTTAAGCTTTACAAAGC-3′, and 5′-GAATTGATGAAAA-CATATGAGC-3′ (this last primer was used in the PCR with F1422A, 1426A and F1422A, 1426A). The resulting plasmid sequences were confirmed by DNA sequencing.

Gene disruptions. The vps10+ locus was disrupted in wild-type Sch. pombe by inserting an internal vps10 gene fragment with Sch. pombe ura4. To amplify vps10 from chromosomal DNA, appropriate oligonucleotides were synthesized and used as PCR primers (Table 1). A 2.0-kb fragment was recovered and ligated to the pGEM-T Easy vector (Promega). A ClaI site within the cloned vps10+ ORF was digested and a 1.6-kb ura4–case variant (Grimm et al., 1988) was

**METHODS**

**Strains, media and genetic methods.** *Escherichia coli* XL-1 Blue (Stratagene) was used for all cloning procedures. Wild-type Sch. pombe strains TP4-5A (h+ leu1 ura4–D18 ade6–M210) and TP4-1D

- **Gene disruptions.** The vps10+ locus was disrupted in wild-type Sch. pombe by inserting an internal vps10 gene fragment with Sch. pombe ura4. To amplify vps10 from chromosomal DNA, appropriate oligonucleotides were synthesized and used as PCR primers (Table 1). A 2.0-kb fragment was recovered and ligated to the pGEM-T Easy vector (Promega). A ClaI site within the cloned vps10+ ORF was digested and a 1.6-kb ura4–case variant (Grimm et al., 1988) was introduced into the plasmid DNA.
Images were captured with a Sensys cooled charge-coupled device under vacuum. Cells were then suspended in 0.8M urea, 8M urea, 5 % (w/v) SDS, 1 mM EDTA, 50 mM Tris/HCl (pH 6.8), 5 % (v/v) β-mercaptoethanol, lysed by vortexing with an equal volume of glass beads for 90 s, and incubated at 65 °C for 20 min. Cell debris was removed by centrifugation for 3 min at 3000 g. Twenty microlitres of crude proteins (equivalent to 2 x 10⁶ cells) were separated by SDS-PAGE and transferred to PVDF filters. Rabbit polyclonal antibody against GFP (Molecular Probes) was used at a 1 : 2000 dilution. Protein-antibody complexes were visualized by chemiluminescence using the Amersham ECL plus system.

**RESULTS**

**Identification of the fission yeast VPS10 homologue**

The budding yeast Vps10p is a type I transmembrane protein, with a 1393 aa N-terminal luminal domain and a cytoplasmic 164 aa C-terminal domain (Fig. 1a). The luminal domain contains two regions (domains 1 and 2) that share 20 % sequence identity. Two cysteine-rich motifs are present in the luminal domain (Jorgensen et al., 1999). An examination of the Sch. pombe genome database revealed a single gene on chromosome II (SPBC16C6.06) predicted to encode a protein homologous to the S. cerevisiae Vps10/Pep1 protein. Therefore, we designated this gene vps10⁺, and vps10⁺ has one intron and encodes a putative transmembrane protein of 1466 aa. The 31 % amino acid identity between the large luminal domains of S. cerevisiae and Sch. pombe Vps10 proteins suggests that SpVps10p is a sorting receptor for vacuolar proteins.

To examine the phenotypic consequences of deleting vps10⁻, we constructed a null allele. A linear fragment of the vps10⁻ gene, into which Sch. pombe ura4 had been inserted, was used to transform haploid strain TP4-1D. Several slow-growing transformants were isolated and the structure of the disrupted allele was verified by Southern blot and PCR analyses (data not shown).

**Disruption of vps10⁺ results in missorting of Cpy1p to the cell surface**

We examined sorting of Sch. pombe CPY (SpCPY) in vps10Δ strains. To confirm missorting of SpCPY to the cell surface in these mutants, we employed a colony blot assay that directly tested cells for secretion of Sch. pombe Cpy1p. In
wild-type cells, Cpy1p is efficiently sorted to the vacuole and is therefore not detected. In contrast, in \textit{vps10}\textsuperscript{D} cells, secretion of SpCPY to the cell surface was observed (Fig. 1b). The rate of Cpy1p maturation was also determined. During the initial 10 min of labelling, the ER- and Golgi-specific precursor form (proCPY) and a small amount of the vacuole-specific mature form (mCPY) were produced in wild-type cells. After a 30 min chase, proCPY was almost completely converted to mCPY (Fig. 1c; Tabuchi \textit{et al.}, 1997a). The \textit{vps10}\textsuperscript{D} mutant exhibited a processing defect for Cpy1p. After a 30 min chase, \textit{vps10}\textsuperscript{D} cells were unable to complete maturation of the majority of proCPY. However, a small amount of mCPY was detected after a 30 min chase in the \textit{vps10}\textsuperscript{D} strain (Fig. 1c). These results indicate that \textit{Sch. pombe} Vps10p is required for delivery of Cpy1p to the vacuole, but also suggest the possibility of a Vps10p-independent mechanism for SpCPY sorting in \textit{Sch. pombe}.

\textbf{Localization of \textit{Sch. pombe} Vps10p}

To determine localization of SpVps10p, we fused GFP to the C terminus of SpVps10p (see Methods). Plasmid-borne, thiamine-repressible Vps10p–GFP complemented the sorting defect of the \textit{vps10}\textsuperscript{Δ} mutant. Cells expressing Vps10p–GFP exhibited punctate fluorescence, suggesting that Vps10p–GFP localized to the Golgi apparatus. To confirm this finding, Vps10–YFP was co-expressed with the Golgi marker protein Gms1–CFP. Gms1p is a UDP-galactose transporter that localizes to the Golgi membrane (Tabuchi \textit{et al.}, 1997b; Tanaka & Takegawa, 2001). Vps10–YFP fluorescence largely co-localized with Gms1–CFP, or the two were observed as adjacent spots of fluorescence (Fig. 2). This indicates that SpVps10p primarily localizes to the Golgi and TGN, in agreement with observations made with \textit{S. cerevisiae} (Marcusson \textit{et al.}, 1994; Cereghino \textit{et al.}, 1995; Piper \textit{et al.}, 1995; Cooper & Stevens, 1996).

\textbf{C-terminal cytoplasmic domain of \textit{Sch. pombe} Vps10p is required for vacuolar sorting of Cpy1p}

Similar in structure to \textit{S. cerevisiae} Vps10p (ScVps10p), SpVps10p appears to be a type I transmembrane protein with 79 aa exposed to the cytoplasm. The tail domain of ScVps10p is divided into two halves, domains 1 and 2. SpVps10p shares similarity to the first half of the cytoplasmic tail that is closest to the transmembrane domain (Fig. 1a).

To examine the functional requirements of the cytoplasmic domain of SpVps10p, a stop codon was inserted at Leu\textsubscript{1408} by site-directed mutagenesis, resulting in truncation of the C-terminal 59 aa. The truncated form of SpVps10p did not
complement the vacuolar protein transport defect in the vps10Δ mutant (Fig. 3a), suggesting that the C-terminal region is crucial for function. This domain is thought to be required for its own localization, because the retromer complex interacts with it to transport Vps10p from the prevacuolar compartment (PVC) to the TGN, as observed in S. cerevisiae (Nothwehr et al., 1999, 2000).

In S. cerevisiae, aromatic amino acid residues have been shown to comprise a PVC-to-TGN retrieval signal, and their alteration affects CPY sorting (Cereghino et al., 1995; Cooper & Stevens, 1996). A di-aromatic motif is found in the cytoplasmic C-terminal domain of the mammalian receptors (Schweizer et al., 1997). SpVps10p possesses F1419SSIPF1426 similar to the tyrosine-based YSSL signal in budding yeast (Cooper & Stevens, 1996). To define sorting signals in SpVps10p that direct transport or recycling of the receptor, F residues were substituted with A by site-directed mutagenesis, and secretion of SpCPY was then examined (Fig. 3b). Secretion of Cpy1p was not detected in vps10Δ cells expressing Vps10p-F1419A or Vps10p-F1425A. However, cells expressing Vps10p-F1426A and doubly substituted Vps10p-F1425, 1426A failed to complement the phenotype of the vps10Δ mutant, indicating the importance of F1426 for SpVps10p function.

Fig. 2. Localization of Vps10p. Wild-type cells containing pAL-nmt41-p-vps10-YFP and pAU-gms1-CFP were cultured in MM-Ura-Leu medium without thiamine for 15 h, and visualized by fluorescence microscopy.

Fig. 3. (a) Truncation of the Vps10p cytoplasmic domain affects receptor function. vps10Δ mutant cells carrying pST1-Δ59 or pST1 were labelled for 10 min and then chased for the indicated times. SpCPY was immunoprecipitated and analysed as described above. The positions of proCPY and mCPY are indicated. (b) F1426A mutation alters Vps10p function. vps10Δ mutant cells containing pST1, pST1-F1419A, pST1-F1425A, pST1-F1426A, or empty vector (V), wild-type cells containing empty vector (WT), cpy1::LEU2 (cpy1Δ), and vps34::LEU2 (vps34Δ) cells were grown on a nitrocellulose filter for 2 days at 30°C and Sch. pombe Cpy1p secretion was detected by immunoblotting. (c) Mutant Vps10p partially localizes to the vacuole. Wild-type cells containing pTN197/vps10 (top), pTN197/vps10-F1419A (middle) or pTN197/vps10-F1425, 1426A (bottom) were grown in MM-Leu medium without thiamine for 24 h and visualized by fluorescence microscopy. Vacuolar membranes are indicated by arrows.
To confirm the cellular localization of mutant Vps10p, Vps10–GFP was subjected to site-directed mutagenesis. Although very little vacuolar Vps10–GFP was observed, a significant amount of mutant Vps10–GFP (F1425, 1426A) was found to localize to the vacuolar membrane in addition to the Golgi/TGN, when the induction period was relatively long (24 h, Fig. 3c). Vps10–F1419A partially localized to the vacuolar membrane, but the remaining proteins were found in the Golgi/TGN, which had the requisite function of Vps10p. This observation suggests that the F1419 and F1425, 1426 to A substitutions affected Vps10p retrieval from the PVC to the Golgi.

**Disruption of Sch. pombe VPS26, VPS29 and VPS35 homologues**

The retromer complex, consisting of 5 vps proteins (Vps5p, Vps17p, Vps26p, Vps29p and Vps35p) is responsible for retrograde transport of ScVps10p (reviewed by Pfeffer, 2001; Seaman, 2005). Three vps proteins, Vps26p, Vps29p and Vps35p, consist of a subcomplex which selects cargo and two other proteins, Vps5p and Vps17p, that comprise a dimer and play a structural role (Nothwehr & Hindes, 1997; Horazdovsky et al., 1997; Seaman et al., 1998; Reddy & Seaman, 2001). The C-terminal cytoplasmic domain of Vps10p interacts with Vps35p on the endosomal membrane, leading to selective sorting into vesicles that are directed back to the Golgi (Nothwehr et al., 1999, 2000). A BLAST search revealed homologous genes in *Sch. pombe* (Table 1; Takegawa et al., 2003a). These genes were cloned and null alleles were constructed. These disruptants all secreted SpVps10p (Fig. 4a), but to a lesser extent than vps10Δ cells. The rate for Cpy1p maturation was also determined. After a 30 min chase, the conversion of proCPY to mCPY appeared slower than in wild-type cells (Fig. 4b). These results strongly suggest that the function and components of the retromer complex are conserved in *Sch. pombe*.

**SpVps10p is mislocalized and unstable in vps26, vps29 and vps35 mutants**

To determine whether the retromer complex is required for correct retention of SpVps10p in the Golgi, Vps10–GFP was expressed in vps5, vps17, vps26, vps29 and vps35 mutants, and cells were stained with FM4-64. FM4-64 is a lipophilic styryl dye used as a vacuolar marker in yeasts (Vida & Emr, 1995, Takegawa et al., 2003a). While Vps10–GFP appeared coincident with Golgi in wild-type cells, Vps10–GFP localized to the vacuolar membrane in vps10 mutants cells (Fig. 5a). After a long incubation (30–48 h), Vps10–GFP exhibited partial vacuolar fluorescence in wild-type cells, while most appeared localized to the Golgi (data not shown). These results indicate that all five vps proteins are required for effective retrieval of SpVps10p from the PVC to the Golgi. By Western blotting, degraded Vps10–GFP was detected in these vps mutants, indicating instability of SpVps10p (Fig. 5b). Vps10–GFP mislocalized to vacuolar membranes, suggesting that the N-terminal domain was degraded by vacuolar proteases.
is dependent solely on SpVps10p. When SpCPY is produced in S. cerevisiae, a small amount of the mature form is detected even in the vps10Δ mutant (Takegawa et al., 2003b). These similar results indicate the presence of an alternative sorting mechanism that may not require a sorting receptor. A candidate is bulk flow of vacuolar protein traffic; however, this remains to be clarified.

As described above, S. cerevisiae Vps10p contributes to maturation of two other vacuolar proteases, PrA and PrB (Whyte & Munro, 2001). Overproduction of PrA results in a great amount of CPY secretion even in the wild-type S. cerevisiae (Westphal et al., 1996). The fission yeast Vps10p might also have substrate specificity toward other vacuolar proteases. We recently found that two serine proteases, Isp6p and Psp3p (Sato et al., 1994; Ladds & Davey, 2000), homologous to budding yeast PrB, were required for CPY maturation and that they localized to the vacuole (K. Takegawa, Y. Kusunoki, N. Tanaka and Y. Giga-Hama, unpublished results). However, CPY secretion was not detected when either Isp6p or Psp3p was overexpressed in wild-type cells (data not shown). In addition, overexpression of these proteases did not increase mis-sorting of CPY in the vps10Δ mutant. These observations strongly suggest that Isp6p and Psp3p do not compete with CPY for binding to SpVps10p, and that SpVps10p is not needed for transport of these proteases. We conclude that these proteases are transported in a Vps10p-independent manner. We are currently trying to identify the cargo proteins whose transport depends on SpVps10p.

We also found that SpVps10p was primarily localized on Golgi or TGN membranes in fission yeast, similar to S. cerevisiae. This observation strongly suggests that the retromer complex is involved in retrieval of SpVps10p from the PVC to the Golgi. As previously reported, all five vps proteins that comprise the retromer complex, Vps5p, Vps17p, Vps26p, Vps29p and Vps35p, are also conserved in Sch. pombe (Takegawa et al., 2003a; Koga et al., 2004). Mislocalization of SpCPY in these mutants occurs to a lesser extent than in vps10Δ cells, as endogenous SpVps10p in these mutants is functional. However, SpVps10p transits through the PVC and is eventually sorted to vacuolar membranes. These vps proteins are also found in humans. The human homologues hVps26, hVps29 and hVps35 are assembled into multimeric complexes that also comprise SNX1 and SNX2, equivalent to yeast Vps5p and Vps17p, respectively (Haft et al., 2000; Edgar & Polak, 2000). These observations support the notion that the retromer complex is conserved and serves related functions in protein trafficking, thereby recycling receptors in Sch. pombe. The sorting defect in the vps10Δ mutant expressing truncated SpVps10p and the phenotype of vps35Δ cells suggest that the C-terminal cytoplasmic domain of SpVps10p interacts with Vps35p, as observed in S. cerevisiae (Nothwehr et al., 1999, 2000). In fission yeast, however, it is still unclear whether these vps proteins associate to make a complex that interacts with SpVps10p.

SpVps10p exhibits 30% identity with ScVps10p, while the similarity of the C-terminal cytoplasmic domain is much lower than that of the N-terminal luminal domain. In addition, these proteins differ in the size of the C-terminal cytoplasmic domain. This domain in Sch. pombe is about half the size of that in S. cerevisiae. The cycling of Vps10p between the TGN and PVC is directed by signals located in the cytoplasmic domain. Half of this region is sufficient for correct sorting of CPY in S. cerevisiae (Cereghino et al.,

![Fig. 5.](http://mic.sgmjournals.org)
suggesting that sorting of Vps10p is not related to the size of the cytoplasmic domain. The signals for cycling are localized to 79 aa in SpVps10p.

In _S. cerevisiae_, both Vps10p and Kex2p possess motifs in their short cytoplasmic C-terminal domain in which certain aromatic residues have been shown to be important for their retrieval to the Golgi (Cereghino _et al._, 1995; Cooper & Stevens, 1996; Wilcox _et al._, 1992). Some are also found in Vth1p and Vth2p. Binding of Vps35p to the cytoplasmic domain of dipeptidyl amino peptidase A also requires two phenylalanine residues, F$_{145}$ and F$_{147}$, although retrieval of two cargo proteins is reported to be mediated by a distinct domain in Vps35p (Nothwehr _et al._, 1993, 2000). Similar to that found in budding yeast, a di-aromatic motif is present in the cytoplasmic C-terminal domain of the mammalian cation-dependent mannose 6-phosphate receptor, preventing the receptor from trafficking to lysosomes (Schweizer _et al._, 1997). The C-terminal cytoplasmic domain of SpVps10p also possesses aromatic amino acid residues, including a di-aromatic motif with a preceding phenylalanine, F$_{1419}$SSIPFF$_{1426}$ similar to the YSSL signal found to be important for PVC-to-TGN retrieval in _S. cerevisiae_ (Cooper & Stevens, 1996).

In the present study, aromatic amino acids in SpVps10p, especially F$_{1426}$ were found to be required for function. However, impairment of localization occurred to a lesser extent than in retromer complex mutants, indicating that mutant Vps10p-F$_{1425,1426}$A could partially recycle back to the TGN. The F$_{1419}$A mutation caused similar localization to that of Vps10p-F$_{1425,1426}$A, suggesting that F$_{1419}$ is also required for sorting (Fig. 3c). However, Vps10p-F$_{1419}$A could complement the phenotypes of the vps10A mutant, as shown by CPY immunoblot analysis (Fig. 3b). The difference between functionalities of Vps10p-F$_{1419}$A and Vps10p-F$_{1425,1426}$A may be caused by the presence of another signal. Vps10p may contain two separate retrieval signals to either enhance its recycling rate or to improve the efficiency of its recycling, as suggested in _S. cerevisiae_ (Cooper & Stevens, 1996). The F$_{1419}$A and F$_{1426}$A mutations might cause structural alterations, thereby affecting the efficacy of another unidentified signal for recycling. The F$_{1419}$A mutation might enhance the efficiency of a second sorting signal, but the F$_{1426}$A mutation may disturb the same signal. Another possible explanation is that overexpression of Vps10p-F$_{1419}$A, resulting from high copy number, suppresses secretion of SpCPY in _vps10A_, because pST1 and the parent vector pALKS+ are multi-copy vectors. At present, it is not known whether these aromatic amino acids mediate direct binding to Vps35p; however, F$_{1419}$ and F$_{1426}$ residues play a major role in the membrane trafficking of SpVps10p. Other aromatic amino acid residues might be involved in this process as a second sorting signal, because the cytosolic domain of SpVps10p possesses two additional F and three additional Y residues. Further characterization of localization signals for the cytosolic domain of SpVps10p will be required to identify the SpVps10p sorting signals that effect receptor recycling.

Recently, the cytoplasmic C-terminal domain of ScVps10p has been shown to interact with mammalian Golgi-associated, gamma adaptin ear containing, ADP-ribosylation factor binding protein (GGA)1, but not with GGA2 (Dennes _et al._, 2002). GGAs are suggested to play a role in trafficking of proteins between the TGN and the vacuole (reviewed by Dell’Angelica & Payne, 2001). Budding yeast also has two GGA genes, and disruption of these genes results in mis-sorting of CPY and in morphologically defective vacuoles (Hirst _et al._, 2000; Dell’Angelica _et al._, 2000; Costaguta _et al._, 2001). The C-terminal cytoplasmic domain of ScVps10p contains several segments similar to the acidic cluster-dileucine-like motifs that are also found in budding yeast, a di-aromatic motif is present in the cytoplasmic C-terminal domain of the mammalian cation-dependent mannose 6-phosphate receptor, preventing the receptor from trafficking to lysosomes (Schweizer _et al._, 1997). The C-terminal cytoplasmic domain of SpVps10p also possesses aromatic amino acid residues, including a di-aromatic motif with a preceding phenylalanine, F$_{1419}$SSIPFF$_{1426}$ similar to the YSSL signal found to be important for PVC-to-TGN retrieval in _S. cerevisiae_ (Cooper & Stevens, 1996).

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**REFERENCES**


