Vacuolar protein sorting receptor in *Schizosaccharomyces pombe*

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The mechanism by which soluble proteins, such as carboxypeptidase Y, reach the vacuole in *Saccharomyces cerevisiae* is very similar to the mechanism of lysosomal protein sorting in mammalian cells. Vps10p is a receptor for transport of soluble vacuolar proteins in *S. cerevisiae*. *vps10⁺*, a gene encoding a homologue of *S. cerevisiae* PEP1/VPS10, has been identified and deleted from the fission yeast *Schizosaccharomyces pombe*. Deletion of the *vps10⁺* gene resulted in missorting and secretion of *Sch. pombe* vacuolar carboxypeptidase Cpy1p, indicating that it is required for targeting Cpy1p to the vacuole. *Sch. pombe* Vps10p (SpVps10p) is a type I transmembrane protein and its C-terminal cytoplasmic tail domain is essential for Cpy1p transport to the vacuole. Cells expressing green fluorescent protein-tagged SpVps10p produced a punctate pattern of fluorescence, indicating that SpVps10p was largely localized in the Golgi compartment. In addition, *Sch. pombe* *vps26⁺*, *vps29⁺* and *vps35⁺*, encoding homologues of the *S. cerevisiae* retromer components VPS26, VPS29 and VPS35, were identified and deleted. Fluorescence microscopy demonstrated that SpVps10p mislocalized to the vacuolar membrane in these mutants. These results indicate that the *vps26⁺*, *vps29⁺* and *vps35⁺* gene products are required for retrograde transport of SpVps10p from the prevacuolar compartment back to the Golgi in *Sch. pombe* cells.

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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 mis-sort and secrete the precursors of soluble vacuolar hydrolases, such as carboxypeptidase Y (CPY) and proteinase A (PrA).

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.

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

the *VPS* gene, *VPS10/PEP1*. *VPS10* encodes a type I transmembrane receptor protein responsible for recognition and targeting of CPY to the vacuole (Marcusson *et al.*, 1994). Precursor CPY can bind to Vps10p in the late Golgi compartment (Marcusson *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 (Cooper & Stevens 1996; Seaman *et al.*, 1997, 1998). In addition, Vps10p carries a tyrosine-based localization signal that is essential both for its steady-state localization to the trans-Golgi network (TGN) and for its function as the CPY receptor (Cereghino *et al.*, 1995; Cooper & Stevens 1996).

We have previously reported the cloning and characterization of the *Sch. pombe* CPY gene (*cpy1*⁺) as a vacuolar protein (Tabuchi *et al.*, 1997a). The C-terminal mature region of the *cpy1*⁺ gene product (Cpy1p), which contains the catalytic region, is highly conserved in other serine carboxypeptidases and shows 54% identity to *S. cerevisiae* CPY. In contrast, the N-terminal pro region of *Sch. pombe* Cpy1p containing the vacuolar sorting signal has fewer identical residues. However, the N-terminal pro segment is sufficient to direct delivery of a Cpy1p–invertase fusion protein to the vacuole (Tabuchi *et al.*, 1997a). Recently, we have shown that *Sch. pombe* Cpy1p is recognized by mechanisms similar to those involved in the intracellular sorting of vacuolar proteins through the ER/Golgi/vacuolar sorting pathway in *S. cerevisiae* (Takegawa *et al.*, 2003b). Interestingly, maturation of Cpy1p is severely inhibited in an *S. cerevisiae* *vps10* mutant, indicating that Vps10p is required for targeting Cpy1p to the vacuole (Takegawa *et al.*, 2003b). This result suggests that *Sch. pombe* should possess Cpy1p receptor-like proteins. Several Vps10p-like proteins have been reported in mammalian cells, such as the low density lipoprotein receptor (Yamasaki *et al.*, 1997; Morwald *et al.*, 1997) and receptor-associated protein-binding protein/sortilin (Peterson *et al.*, 1997). Conservation of Vps10p/CPY receptor-like proteins between organisms as widely divergent as yeast and mammals indicates that these proteins play a fundamental role in intracellular protein trafficking. A BLAST search of protein databases for homologues of *S. cerevisiae* Vps10 revealed a single *Sch. pombe* gene on chromosome II (SPBC16C6.06).

In this report, we characterize the fission yeast homologue of the Vps10p/CPY receptor. Deletion of *vps10*⁺ resulted in missorting of *Sch. pombe* vacuolar carboxypeptidase Cpy1p. The fluorescence pattern of green fluorescent protein (GFP)-tagged Vps10p revealed localization to the Golgi and TGN, and showed that *vps* proteins that constitute the retromer complex, all of which are conserved in *Sch. pombe*, are required for correct localization and stability of Vps10p.

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

(*h*⁺ *leu1 ura4–D18 his2 ura4 ade6–M216*) (Ohkura *et al.*, 1989) were obtained from Dr T. Toda (Cancer Research UK, London Research Institute), and KJ100-7B (*h*⁹⁰ *leu1 ura4*) was obtained from Dr K. Tanaka (Tokyo University, Japan). *cpy1* (*h*⁺ *leu1-32 his2 ura4–D18 ade6–M216 cpy1::ura4*⁺) and *vps34/pik3* (*h*⁺ *leu1-32 ura4–D18 ade6–M216 vps34::ura4*⁺) mutants were constructed as described by Tabuchi *et al.* (1997a) and Takegawa *et al.* (1995). The *vps5Δ* (*h*⁹⁰ *leu1 ura4–D18 ade6–M210 vps5::ura4*⁺) (Koga *et al.*, 2004), *vps17Δ* (*h*⁹⁰ *leu1 ura4–D18 ade6–M210 his2 vps17::ura4*⁺) (Koga *et al.*, 2004) and *vps35Δ* (*h*⁹⁰ *leu1 ura4–D18 ade6–M210 vps35::ura4*⁺) mutants were obtained from Dr Y. Fukui (Tokyo University). Standard rich medium containing YES (5 g l^{−1} yeast extract, and 30 g l^{−1} glucose) or YPD (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′-CGATATGCTGATTACTAGTGATTTTCAGT-3′ was used to replace leucine₁₄₀₈ with a stop codon and introduce the *SpeI* site (underlined), resulting in pST1-Δ59.

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 SpVps10–YFP, the *vps10*⁺ ORF, under control of the attenuated *nmt1* promoter, was amplified from pTN197/*vps10* and subcloned into the *Apal* and *SmaI* sites of pAL-KS⁺, then YFP from pEYFP (BD Biosciences Clontech) was inserted into the *SmaI* and *NotI* sites, resulting in pAL-*nmt41p-vps10*-YFP. To tag Gms1p with CFP, a 1.3 kb *SmaI*–*BamHI* fragment encoding *gms1*⁺ and its promoter was subcloned into pAU-KS⁺. A *BamHI*–*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′-TGCTTCATCAATTCCAATATTTCTTTTCCGC-3′ and 5′-GCTCCTCTTGTTGACGATATGCCTGATTCC-3′; F₁₄₂₅ with A (F₁₄₂₅A), 5′-CAATAGCTTTTCCGCTCTTTACCAAA-GCG-3′; F₁₄₂₆ with A (F₁₄₂₆A), 5′-CAATATTCGCTTCCGCTCTTTACCAAGCG-3′; F₁₄₂₅ and F₁₄₂₆ with A (F₁₄₂₅, 1426A), 5′-CAATAGCTGCTTCCGCTCTTTACCAAGCG-3′ and 5′-GAATTGATGAAAAAGCTCCTCTTGTTGACG-3′ (this last primer was used in the PCR with F₁₄₂₅A, F₁₄₂₆A and F₁₄₂₅, 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*⁺ cassette (Grimm *et al.*, 1988) was

Table 1. PCR primers used to amplify the *Sch. pombe* genes analysed in this study

Gene	Homologue* (E-value)	Primers (5'–3')	Restriction enzyme†
<i>vps26</i> ⁺	VPS26 (4·7e-69)	5'-TACTAGCTCTTCACAGTATCACCTACACC-3' 5'-GTCTCGAAATGTTGGTGTAAAGCGTAGCC-3'	<i>Clal</i>
<i>vps29</i> ⁺	VPS29 (2·0e-37)	5'-CTTCCCCTCAATATGAATCTGAG-3' 5'-CTCTATCACAAGAGATTCTGCG-3'	<i>XhoI</i> <i>HindIII</i>
<i>vps10</i> ⁺	VPS10 (5·5e-136)	5'-CTCTTTTCTAAATGCTGAATTTGGTGACG-3' 5'-GAAATGTGTTTTCCTCAGGAACCG-3'	<i>Clal</i>

*The most similar *S. cerevisiae* homologues are listed with probability scores (E-values) shown in parentheses.

†Cloned PCR products were digested with the indicated restriction enzymes in order to construct gene disruptions through insertion of *ura4*⁺ as a selectable marker.

inserted. A linearized DNA fragment carrying the disrupted *vps10* gene was used to transform wild-type haploid strains TP4-1D (*h*⁺) and KJ100-7B (*h*⁹⁰) and *ura*⁺ transformants were selected. To confirm that one of the *vps10* genes had been disrupted, *ura*⁺ transformants were analysed by Southern blotting and PCR to verify correct integration of the deletion constructs.

vps26⁺ (SPAC4G9.13c), *vps29*⁺ (SPAC15E1.06) and *mrl1*⁺ (SPBC530.09c) were amplified by PCR using appropriate primers and cloned into the pGEM-T EASY vector. The genes were disrupted by replacing coding sequences with *ura4*, using restriction sites as summarized in Table 1. The linearized DNA fragments carrying these disrupted genes were then used to transform wild-type haploid strains TP4-1D and KJ100-7B. To confirm that the genes had been disrupted, *ura*⁺ transformants were analysed by Southern blotting and PCR to verify correct integration of the deletion constructs.

Fluorescence microscopy. Vacuoles within wild-type and mutant fission yeast were labelled with FM4-64 (Iwaki *et al.*, 2003). Cells were examined with an Olympus BX-60 fluorescence microscope using a U-MGFPHQ filter (for GFP), a U-MCFPHQ filter (for CFP), a U-MYFPHQ filter (for YFP) or a U-MWIG filter (for FM4-64). Images were captured with a Sensys cooled charge-coupled device (CCD) camera using MetaMorph (Roper Scientific) and saved as Adobe Photoshop files on a Macintosh G4 computer.

Pulse-chase analysis of the *Sch. pombe* Cpy1 protein. Pulse-chase analysis and immunoprecipitation of the vacuolar CPY from *Sch. pombe* were carried out as described by Tabuchi *et al.* (1997a). Antibody incubations were carried out using rabbit polyclonal antibody against *Sch. pombe* Cpy1p (Tabuchi *et al.*, 1997a).

Immunoblot analysis. Immunoblot analysis to detect mislocalized Cpy1p was performed as described by Cheng *et al.* (2002). Briefly, cells were spotted on nitrocellulose membranes and grown for 2 days at 30 °C. After removing cells by washing, nitrocellulose membranes were subjected to immunodetection of Cpy1p using rabbit polyclonal antibodies against *Sch. pombe* Cpy1p (1:500 dilution), horseradish peroxidase-conjugated anti-rabbit IgG antiserum (Amersham Biosciences), and the Amersham ECL system.

For Western blot analysis of SpVps10–GFP, crude protein extracts were prepared. Cells were grown in MM medium without leucine (MM-Leu) for ~20 h, and 10⁸ cells were harvested and washed once with distilled water. Cells were resuspended in 0·3 ml 10% (w/v) TCA, incubated on ice for 1 h, and precipitated by centrifugation for 10 min at 20 000 g at 4 °C. Cells were washed twice with acetone and dried under vacuum. Cells were then suspended in 0·1 ml cracking buffer [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 × 10⁷ 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*⁺. *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

Fig. 1. (a) Predicted structures of ScVps10p and SpVps10p. The N-terminal luminal portion contains two putative domains, 1 and 2, each with a cysteine-rich region. The C-terminal cytoplasmic tail is also divided into two halves, indicated as 1 and 2. Transmembrane segments are described in GeneDB (<http://www.genedb.org/genedb/>) and the *Saccharomyces* Genome Database (<http://genome-www.stanford.edu/Saccharomyces/>). (b) Immunoblot analysis of *Sch. pombe* Cpy1p. Cells were grown on a nitrocellulose filter for 2 days at 30 °C and the filter was processed for immunoblotting using rabbit polyclonal antibody against *Sch. pombe* Cpy1p. *cpy1Δ*, negative control; *vps34Δ*, positive control. (c) *In vivo* processing of *Sch. pombe* Cpy1p. Wild-type (WT) and *vps10Δ* cells were pulse-labelled with Express³⁵S (NEN Research Products, Boston, MA) for 10 min at 28 °C and then chased for the indicated times. The immunoprecipitates were separated on an SDS-10% polyacrylamide gel. The autoradiograms of the fixed, dried gels are shown. The positions of proCPY (110 kDa) and mCPY (32 kDa) are indicated.

wild-type cells, Cpy1p is efficiently sorted to the vacuole and is therefore not detected. In contrast, in *vps10Δ* 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 *et al.*, 1997a). The *vps10Δ* mutant exhibited a processing defect for Cpy1p. After a 30 min chase, *vps10Δ* 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 *vps10Δ* strain (Fig. 1c). These results indicate that *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 *Sch. pombe*.

Localization of *Sch. pombe* Vps10p

To determine localization of SpVps10p, we fused GFP to the C terminus of SpVps10p (see Methods). Plasmid-borne, thiamine-repressible Vps10–GFP complemented the sorting defect of the *vps10Δ* mutant. Cells expressing Vps10–GFP exhibited punctate fluorescence, suggesting that Vps10–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 *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 *S. cerevisiae* (Marcusson *et al.*, 1994; Cereghino *et al.*, 1995; Piper *et al.*, 1995; Cooper & Stevens, 1996).

C-terminal cytoplasmic domain of *Sch. pombe* Vps10p is required for vacuolar sorting of Cpy1p

Similar in structure to *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 Leu₁₄₀₈ by site-directed mutagenesis, resulting in truncation of the C-terminal 59 aa. The truncated form of SpVps10p did not

Fig. 2. Localization of Vps10p. Wild-type cells containing pAL-*nmf41p-vps10*-YFP and pAU-*gms1*-CFP were cultured in MM-Ura-Leu medium without thiamine for 15 h, and visualized by fluorescence microscopy.

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 F₁₄₁₉SSIIFF₁₄₂₆ 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-F_{1419A} or Vps10p-F_{1425A}. However, cells expressing Vps10p-F_{1426A} and doubly substituted Vps10p-F_{1425,1426A} failed to complement the phenotype of the *vps10Δ* mutant, indicating the importance of F₁₄₂₆ for SpVps10p function.

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) *F*_{1426A} mutation alters Vps10p function. *vps10Δ* mutant cells containing pST1, pST1-F_{1419A}, pST1-F_{1425A}, pST1-F_{1426A}, pST1-F_{1425,1426A}, 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*-F_{1419A} (middle) or pTN197/*vps10*-F_{1425,1426A} (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 SpCPY (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 *vps* mutant 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.

Fig. 4. (a) CPY secretion in *vps26*, *vps29* and *vps35* mutants. The indicated strains were grown on a nitrocellulose filter for 2 days at 30 °C and the filter was processed for immunoblotting using rabbit polyclonal antibody against *Sch. pombe* Cpy1p. (b) Vacuolar transport of CPY in *vps26*, *vps29* and *vps35* mutants. The indicated strains were pulse-labelled with Express³⁵S for 15 min at 30 °C and chased for the indicated times. CPY was immunoprecipitated and analysed as described above.

DISCUSSION

We have identified the *Sch. pombe* *vps10*⁺ gene homologous to *S. cerevisiae* VPS10. A search of the *Sch. pombe* genome database indicates that there are no additional *S. cerevisiae* VPS10 homologues. This is intriguing because *S. cerevisiae* has two VPS10 homologues, VTH1 and VTH2, and both Vth1p and Vth2p are capable of sorting CPY and PrA (Cooper & Stevens, 1996).

Consistent with observations made in *S. cerevisiae* (Marcusson *et al.*, 1994; Cooper & Stevens, 1996), fission yeast *vps10Δ* cells were found to secrete CPY and to exhibit a severe defect in CPY maturation. These phenotypes are consistent with the predicted SpVps10p function as a sorting receptor that binds CPY in the late Golgi and then specifically transports it to the vacuole. However, a small fraction of mature CPY was detected, indicating that some CPY was delivered to vacuoles. We postulate that CPY maturation in *vps10Δ* may be mediated by the mannose 6-phosphate receptor homologue Mrl1p (SPBC530.09c). In budding yeast, it was recently reported that Mrl1p and Vps10p make overlapping contributions to the delivery and maturation of PrA and proteinase B (PrB), although targeting of CPY appears to be dependent on Vps10p alone (Whyte & Munro, 2001). In fission yeast, sorting of CPY was not affected substantially by deletion of *mrl1*. The effect of the *vps10* deletion was unchanged by additional deletion of *mrl1* (data not shown). Thus, we conclude that CPY maturation in fission yeast cells

Fig. 5. (a) Localization of Vps10-GFP in *vps26*, *vps29* and *vps35* mutants. Cells containing pTN197/*vps10* were grown in MM-Leu medium without thiamine for 20 h, then labelled with FM4-64 for 30 min and chased for 90 min at 30 °C. Vps10-GFP (left), vacuoles that label with FM4-64 (centre) and Nomarski optics (right) are indicated. (b) Western blotting analysis of Vps10-GFP. Crude proteins were subjected to SDS-PAGE, transferred to a PVDF filter, and processed for immunoblotting using rabbit polyclonal antibody against GFP.

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 missorting 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.*,

1995), 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, F₈₅ and F₈₇, 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, F₁₄₁₉SSIPIFF₁₄₂₆, 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 F₁₄₂₆, 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-F₁₄₂₅, ₁₄₂₆A could partially recycle back to the TGN. The F₁₄₁₉A mutation caused similar localization to that of Vps10p-F₁₄₂₅, ₁₄₂₆A, suggesting that F₁₄₁₉ is also required for sorting (Fig. 3c). However, Vps10-F₁₄₁₉A could complement the phenotypes of the *vps10Δ* mutant, as shown by CPY immunoblot analysis (Fig. 3b). The difference between functionalities of Vps10-F₁₄₁₉A and Vps10p-F₁₄₂₅, ₁₄₂₆A 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 F₁₄₁₉A and F₁₄₂₆A mutations might cause structural alterations, thereby affecting the efficacy of another unidentified signal for recycling. The F₁₄₁₉A mutation might enhance the efficiency of a second sorting signal, but the F₁₄₂₆A mutation may disturb the same signal. Another possible explanation is that overexpression of Vps10-F₁₄₁₉A, resulting from high copy number, suppresses secretion of SpCPY in *vps10Δ*, 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, F₁₄₁₉ and F₁₄₂₆ 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 serve as GGA-binding sites in sortilin and in mannose 6-phosphate receptors (MPRs) (Dell'Angelica & Payne, 2001). SpVps10p has a potential dileucine-like motif, E₁₄₄₈NAAFL₁₄₅₃, although this region has no obvious acidic cluster. The C-terminal portion of SpVps10p contains negatively charged residues D₁₄₅₉DDDEE₁₄₆₄, but has SV₁₄₆₆ instead of LL. These features may provide a useful tool in future efforts to identify sorting signals.

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