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Vacuolar Protein Sorting in Fission Yeast: Cloning, Biosynthesis, Transport, and Processing of Carboxypeptidase Y from *Schizosaccharomyces pombe*

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PCR was used to isolate a carboxypeptidase Y (CPY) homolog gene from the fission yeast *Schizosaccharomyces pombe*. The cloned *S. pombe cpy1*$^+$ gene has a single open reading frame, which encodes 950 amino acids with one potential N-glycosylation site. It appears to be synthesized as an inactive pre-pro protein that likely undergoes processing following translocation into appropriate intracellular organelles. The C-terminal mature region is highly conserved in other serine carboxypeptidases. In contrast, the N-terminal pro region containing the vacuolar sorting signal in CPY from *Saccharomyces cerevisiae* shows fewer identical residues. The pro region contains two unusual repeating sequences; repeating sequence I consists of seven contiguous repeating segments of 13 amino acids each, and repeating sequence II consists of seven contiguous repeating segments of 9 amino acids each. Pulse-chase radiolabeling analysis revealed that Cpy1p was initially synthesized in a 110-kDa pro-precursor form and via the 51-kDa single-polypeptide-chain intermediate form which has had its pro segment removed is finally converted to a heterodimer, the mature form, which is detected as a 32-kDa protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Like *S. cerevisiae* CPY, *S. pombe* Cpy1p does not require the N-linked oligosaccharide moiety for vacuolar delivery. To investigate the vacuolar sorting signal of *S. pombe* Cpy1p, we have constructed *cpy1*$^+$*-SUC2* gene fusions that direct the synthesis of hybrid proteins consisting of N-terminal segments of various lengths of *S. pombe* Cpy1p fused to the secreted enzyme *S. cerevisiae* invertase. The N-terminal 478 amino acids of Cpy1 are sufficient to direct delivery of a Cpy1-Inv hybrid protein to the vacuole. These results showed that the pro peptide of Cpy1 contains the putative vacuolar sorting signal.

The budding yeast *Saccharomyces cerevisiae* has served as a model for investigating the mechanism of sorting newly synthesized proteins to intracellular organelles in eukaryotic cells. The protein sorting to the vacuole that is analogous to the mammalian lysosome is one of the best-characterized protein localization processes in *S. cerevisiae*. Proteins that are destined for the vacuole transit the early stages of the secretory pathway (27, 41) before being sorted in a late Golgi compartment away from proteins destined for the cell surface (16). Unlike the mammalian lysosomal sorting system, the carbohydrate moiety does not serve as the sorting signal in *S. cerevisiae*. The information for the sorting of these vacuolar hydrolases appears to be contained within the polypeptide chains themselves (21, 26, 60). The most thoroughly studied soluble vacuolar protein in *S. cerevisiae* is carboxypeptidase Y (CPY). The vacuole sorting signal of *S. cerevisiae* CPY has been defined and shown to reside in a short stretch of amino acids at the N terminus of its prosegment QRPL (61), and the CPY is sorted and targeted to the vacuole in the late Golgi compartment by Vps10p/Pep1p, the CPY sorting receptor that recognizes this vacuolar sorting signal (31, 62).

Several genetic selection studies that used CPY as a vacuolar protein marker in *S. cerevisiae* identified a large number of mutants defective in the delivery of protein to the vacuole. Analyses of the products of these *VPS* (vacuolar protein sorting) genes have revealed numerous molecules whose biochemical activities suggest that they represent important factors regulating vacuolar protein localization (3, 22, 41, 42, 44). Recently it has been found that some homologs of these *VPS* genes exist in other eukaryotic cells, such as those of mammals, plants, molds, and fission yeast (59, 63, 65, 68). It is suggested that the vacuole or lysosome protein delivery system is conserved in all eukaryotic cells. Some of the *VPS* genes encode functionally unknown proteins, although these genes have been sequenced. Therefore, few vacuole or lysosome protein delivery systems are well understood.

The fission yeast *Schizosaccharomyces pombe*, taxonomically and evolutionarily distant from the budding yeast (45), is genetically and physiologically well characterized (12, 33). Some features such as cell cycle, chromosome structure, and RNA splicing are more similar between mammalian cells and *S. pombe* than between mammalian cells and *S. cerevisiae* (34). However, little is known about the protein sorting system in *S. pombe*. Recently, expression of CPY from *S. cerevisiae* in *S. pombe* was reported (52), demonstrating that *S. cerevisiae* CPY is sorted to the vacuole in *S. pombe*. However, there are no well-characterized vacuolar proteins in *S. pombe*, and a specific *S. pombe* vacuolar protein has yet to be identified.

In this paper, we report the identification and characterization of a gene from the fission yeast *S. pombe*, *cpy1*$^+$, which encodes a CPY homolog. The pro region of the *cpy1*$^+$ gene product is larger than that from other yeasts and contains two unusual repeating sequences. Unlike *S. cerevisiae* CPY, *S.*

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TABLE 1. *S. pombe* strains used in this study

Strain	Genotype	Source or reference
TP4-1D	*h*$^+$ *leu1 his2 ura4 ade6-M216*	Ohkura et al. (38)
TP4-5A	*h*$^-$ *leu1 ura4 ade6-M210*	Ohkura et al. (38)
MTD2	*h*$^+$ *leu1 his2 ura4 ade6-M216 cpy1::ura4*$^+$	This study
MTD3	*h*$^+$ *leu1 his2 ura4 ade6-M216 cpy1::LEU2*	This study
NOD1	*h*$^+$ *leu1 his2 ura4 ade6-M216 inv1*	Unpublished data
JSD1	*h*$^+$ *leu1 his2 ura4 ade6-M216 inv1 vps34::LEU2*	Unpublished data

pombe Cpy1p is a dimeric serine carboxypeptidase like wheat carboxypeptidase II and human protective protein/cathepsin A. Through the study of hybrid proteins, consisting of N-terminal segments of Cpy1p fused to the secretory enzyme *S. cerevisiae* invertase, it was demonstrated that the N-terminal 478 amino acids of Cpy1p are sufficient to direct delivery of the Cpy1-Inv hybrid protein to the vacuole. Our results suggest that the vacuole sorting signal of *S. pombe* Cpy1p is contained in the pro segment of Cpy1p, as is the case for *S. cerevisiae* CPY.

MATERIALS AND METHODS

Strains, media, and genetic methods. *Escherichia coli* XL1-blue (Stratagene, La Jolla, Calif.) was used for all cloning procedures. *S. pombe* strains used in this study are listed in Table 1. Standard rich medium (yeast extract-peptone-dextrose) and synthetic minimal medium (MM) for *S. pombe* cells were used as described elsewhere (34), and for pulse-chase analysis, cells were grown in SC medium (43). *S. pombe* cells were transformed by the lithium acetate method as described previously (39). The general genetic methods that were used have been described previously (1).

Reagents. DNA restriction and modifying enzymes were from either Takara Shuzo (Kyoto, Japan) or New England Biolabs (Beverly, Mass.). Zymolyase 100T was obtained from Seikagaku Kogyo (Tokyo, Japan). Novozym 234 was obtained from Novo Nordisk. Deoxynucleotides and the Flexiprep kit were products of Pharmacia. Express^{35}S-label was from ICN (Irvine, Calif.). All other chemicals were from Sigma Chemical (St. Louis, Mo.).

PCR amplification and cloning of the *cpy1*$^+$ gene from *S. pombe*. To amplify CPY-like sequences from cDNA libraries of *S. pombe*, the following oligonucleotides were synthesized: 1, 5′-GTTTGAATTCGG(A/T/G/C)GA(A/G)(A/T)(G/C)(A/T/G/C)TA(T/C)GC(A/T/G/C)GG-3′; 2, 5′-GTTTGGATCC(A/T/G/C)A(A/C)CCA(A/G)TT(A/G)CA(A/T/G)AT-3′; and 3, 5′-GTTTGGATCCA(A/T)(A/T/G/C)GG(A/T/G/C)ACCAT(A/G)TG(A/T/G/C)AT-3′. To facilitate cloning of the PCR products after amplification, *Bam*HI and *Eco*RI restriction sites were incorporated at the 5′ ends of the oligonucleotides. Oligonucleotide 1 encodes amino acids GESYAG, oligonucleotide 2 encodes ICNWLG, and oligonucleotide 3 encodes GGHMVP. The PCR mixture (46) contained 3 μg of *S. pombe* cDNA library, 0.5 μg of primers, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphates, and 5 U of TakaraTaq DNA polymerase (Takara Shuzo Co.) in a total volume of 50 μl. The thermocycler was programmed to complete 30 cycles with the following protocol: 94°C for 1 min, 51°C for 1.5 min, and 72°C for 1.5 min. The reaction product was digested with *Bam*HI and *Eco*RI and resolved by electrophoresis in a 1.5% agarose gel. The product was eluted from the gel and ligated into a pBluescript KS$^-$ vector that had been digested with *Bam*HI and *Eco*RI. The cloned PCR fragment was sequenced with an A.L.F. DNA sequencer (Pharmacia).

S. pombe chromosomal DNA was isolated as described elsewhere (1). The DNA was partially digested with *Sau*3AI, and fragments of 8 to 15 kb were purified with a Takara EASY Trap Kit and ligated into *Bam*HI-digested λEMBL3. This was packaged into phage particles and used to infect *E. coli* P2392. The entire *cpy1*$^+$ gene was isolated by plaque hybridizing the cloned PCR product, labeled with an enhanced chemiluminescence random prime kit (Amersham).

Disruption of the *cpy1*$^+$ gene. The *cpy1*$^+$ locus was disrupted in the TP4-1D wild-type *S. pombe* strain by replacing an internal *cpy1*$^+$ gene fragment with the *S. pombe ura4*$^+$ gene to produce strain MTD-2. A 0.3-kb *Eco*RI-*Xba*I fragment was eliminated from the cloned *cpy1*$^+$ open reading frame, and a 1.6-kb *ura4*$^+$ cassette (17) was inserted. A linearized DNA fragment carrying this disrupted *cpy1*$^+$ gene was used to transform a wild-type haploid strain. To confirm that the *cpy1*$^+$ gene had been disrupted, *ura*$^+$ transformants were analyzed by Southern blot to verify correct integration of the deletion constructs.

Plasmid constructions and oligonucleotide-directed mutagenesis. Conventional recombinant DNA methods were used in the construction and propagation of all plasmids (47). The yeast shuttle vectors pAL-KS$^+$ and pAU-SK$^+$ were kindly provided by C. Shimoda (Osaka City University). Oligonucleotide-directed mutagenesis of the *cpy1*$^+$ gene was performed with *dut ung* mutant *E. coli* as described previously (28). The *cpy1*$^+$-containing plasmids were introduced into the *dut ung* mutant *E. coli* strain CJ236, and single-stranded plasmid DNA was isolated and mutagenized as described in the Takara Mutan K kit manual.

pMT1 and pMT2 were constructed by inserting the subcloned 4.1-kb *Sca*I-*Xba*I genomic DNA fragment of the *cpy1*$^+$ gene into the *Eco*RV-*Xba*I site present in pAL KS$^+$ and pAU SK$^+$, respectively. The single-stranded pMT2 plasmid derived from bacteriophage M13mp18 was used as a template for oligonucleotide-directed mutagenesis. Oligonucleotide TPM7 (T_{1713}GGTCCTGG TACCTCTTCCCTT$_{1734}$) was used for construction; Cys^{575}, predicted to form a disulfide bridge with Cys^{828} on Cpy1, was replaced with Thr. In oligonucleotide TPM6 (C_{1815}TCCAATGCTGCAGTTATCTTT$_{1835}$), also used for construction, a serine residue is substituted for alanine in Asn^{607}-Ala-Ser.

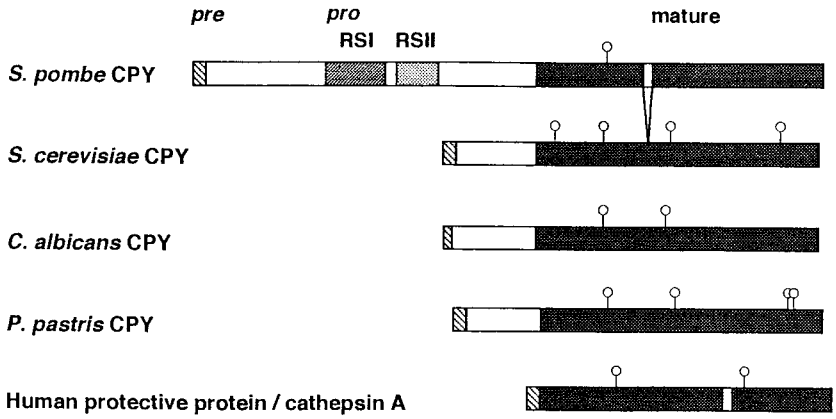
The *cpy1*$^+$*-SUC2* gene fusions were constructed as follows: oligonucleotide-directed mutagenesis of the *cpy1*$^+$ gene was used to insert *Bam*HI sites immediately after the coding sequence for Cpy1 amino acids 8, 23, 194, 376, 478, and 905. pBS-SEYC306 was constructed by inserting a 2.5-kb *Bam*HI-filled *Sal*I restriction fragment containing a truncated *SUC2* gene derived from pSEYC306 (21) into the *Bam*HI-filled *Xba*I sites present in pBluescript SK$^-$. The *Bam*HI-*Not*I restriction fragment containing a truncated *SUC2* gene derived from pBS-SEYC306 was then cloned into the *Bam*HI-*Not*I site present in each mutated pMT1 plasmid to generate plasmids pCpy1^{N8}-Inv, pCpy1^{N23}-Inv, pCpy1^{N194}-Inv, pCpy1^{N376}-Inv, pCpy1^{N478}-Inv, and pCpy1^{N905}-Inv.

Preparation of *S. pombe* Cpy1p-specific antiserum. A fusion between the *E. coli trpE* gene and the *cpy1*$^+$ gene was constructed by subcloning a 648-bp PCR fragment encoding amino acids 661 to 876 of *S. pombe* CPY into *Bam*HI-*Sma*I-digested pATH1 (9), generating an in-frame fusion gene. Induction of the *trpE-cpy1* fusion protein was accomplished as previously described by Kleid et al. (25). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a prominent 55-kDa band representing the fusion protein which was excised and electroeluted from the gel slices with an Atto AE-3590 electrochamber. Approximately 1 mg of the fusion protein was emulsified with Freund's complete adjuvant and injected intramuscularly and subcutaneously into a young male New Zealand White rabbit. Antiserum was collected and screened by immunoprecipitation.

SDS-PAGE and Western blotting (immunoblotting). Cells grown in MM were harvested in the mid-log phase, washed twice with distilled water, and suspended in 50 mM sodium acetate–2 mM EDTA, pH 4.8. Acid-washed glass beads with a volume approximately equal to that of the packed cell pellet were added to the cell suspension, and the cells were disrupted by 10 cycles of vortex mixing for 1 min and standing on ice for 1 min. For nonreducing conditions, an equal volume of the native sample buffer (40% glycerol with 0.1% bromophenol blue) was added to the lysate. For reducing conditions, an equal volume of the denatured sample buffer (4% SDS, 40% glycerol, 3% 2-mercaptoethanol, 0.1% bromophenol blue, 0.1 M Tris-HCl, pH 6.8) was added to the lysate and incubated at 100°C for 5 min. The mixtures were subjected to SDS-PAGE (30), and the separated proteins in the gel were transferred electrophoretically to an Immobilon-P transfer membrane (Millipore). The sheet was blocked with 10% nonfat dry milk at 4°C overnight and probed with rabbit antiserum against the *trpE-cpy1* fusion protein. The protein was then detected with horseradish peroxidase-conjugated antibody against rabbit immunoglobulin G (Amersham).

Cell labeling and immunoprecipitation of the *S. pombe* Cpy1 protein. For analysis of *S. pombe* Cpy1p, *S. pombe* cells were grown to mid-log phase in SC medium at 28°C with the appropriate amino acids. Five optical density equivalents at 600 nm of a yeast culture were collected by centrifugation and resuspended in 0.2 ml of SC medium containing 1 mg of bovine serum albumin per ml and 100 μCi of Express^{35}S-label. The cells were labeled and chased with excess unlabeled methionine and cysteine. The labeling-chase reactions were terminated by the addition of an equal volume of cold 2× stop buffer (2 M sorbitol, 50 mM Tris-HCl [pH 7.5], 40 mM NaF, 40 mM NaN_3, and 20 mM dithiothreitol), and the cultures were incubated on ice for 5 min. Zymolyase 100T and Novozym 234 were added at 80 μg/ml each, and the cells were incubated at 30°C for 25 min. The proteins were precipitated by the addition of trichloroacetic acid to a final concentration of 5%. Cells were centrifuged and washed with acetone twice before lysis. Cells were lysed by vortexing in the presence of glass beads, and protein was solubilized by incubation in a boiling buffer at 100°C for 4 min. Lysates were cleaned by centrifugation, the supernatant was diluted 10-fold into immunoprecipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Tween 20, 0.1 mM EDTA), anti-*S. pombe* Cpy1 antiserum was added, and this solution was incubated at 4°C overnight with constant shaking. Protein A-Sepharose (Pharmacia) was added to the solution and incubated for 2 h in order to collect the antigen-antibody complexes. The protein A-Sepharose–antigen-antibody complexes were centrifuged and washed twice with immunoprecipitation buffer and twice with this solution containing 2 M urea. Then, antibody and immunoprecipitated protein were dissociated by incubation in SDS-PAGE solubilization buffer at 100°C for 4 min. The labeled, immunoprecipitated protein was analyzed by SDS-PAGE–fluorography as previously described (7).

Vacuole preparation. Vacuoles were prepared as described by Nishikawa et al. (36). Cells were grown to an optical density at 600 nm (OD_{600}) of 0.5 to 1.0 in 1 liter of MM, harvested by centrifugation, washed twice with distilled water, and resuspended at 100 OD_{600} units/ml in 10 mM Tris-HCl (pH 7.5) plus 1 M sorbitol. To this suspension, 500 μg of Zymolyase 20T and 500 μg of Novozyme



S. pombe CPY vs.	*pro*	mature
S. cerevisiae CPY	—	54.4
C. albicans CPY	—	57.3
P. pastris CPY	—	56.9
Human protective protein / cathepsin A	—	30.9

FIG. 1. Comparison of *S. pombe* Cpy1 with related serine carboxypeptidases. Human protective protein/cathepsin A is a human CPY homolog protein, which is a dimeric serine carboxypeptidase. The pre region, pro region, and mature region are indicated. The pro region of *S. pombe* Cpy1 contains repeating sequences I and II (RSI and RSII, respectively). The bottom half of the figure is the percentage similarity based on the default amino acid comparison table.

234 were added, and the mixture was incubated at 30°C for 60 min. Spheroplasts were collected by centrifugation and washed twice with 1 M sorbitol. Further procedures were all carried out on ice. The spheroplast pellet was suspended at 100 OD_{600} units/ml in 10 mM morpholineethanesulfonic acid (MES)-Tris (pH 6.9)–0.1 mM $MgCl_2$–12% Ficoll 400 and homogenized by 10 strokes in a Dounce homogenizer. The homogenate was placed on the cushion of 1 ml of 20% Ficoll in a Hitachi 13PA tube and was successively overlaid with 2 ml spheroplast lysate, 3 ml of 12% Ficoll 400 in 10 mM MES-Tris (pH 6.9)–0.1 mM $MgCl_2$, 2 ml of 8% Ficoll 400 in 10 mM MES-Tris (pH 6.9)–0.1 mM $MgCl_2$–0.2 M NaCl, and 2 ml of 0.2 M sorbitol in 10 mM MES-Tris (pH 6.9)–0.5 mM $MgCl_2$–0.2 M NaCl. The discontinuous Ficoll gradient was centrifuged in an RPS-40T rotor (Hitachi) at 100,000 × *g* at 4°C for 10 h. Fractions of 0.5 ml were collected. Vacuoles were collected from the 8 to 0% Ficoll interface.

Enzyme assays. Carboxypeptidase activities from *S. pombe* cells were measured as described by Stevens et al. (58). *S. pombe* cells were grown to mid-log phase in MM and precipitated by centrifugation. Cells were washed in 0.2 M phosphate buffer, pH 7.0, and lysed in the presence of 1% Triton X-100. The cells were suspended in 0.2 ml of 5 mM *N*-carbobenzoxy-L-phenylalanyl-L-leucine (CBZ-Phe-Leu) and incubated at 30°C for 30 min. The reaction was stopped by boiling for 3 min, and released leucine was measured by the addition of 1 ml of 0.2 M phosphate buffer containing 0.15 mg of L-amino oxidase per ml, 10 μg of horseradish peroxidase per ml, 0.5 mM *N*-ethylmaleimide, and 0.3 mg of *O*-dianisidine per ml. After 30 min at 37°C, 1 ml of 6 N HCl was added, and the sample was centrifuged. The A_{540} of the supernatant solution was recorded.

A plate-staining procedure for carboxypeptidase activity was used to screen for a *cpy1*Δ strain (23). Invertase liquid and plate assays were done as described previously (21, 40), and the activity of glucose-6-phosphate dehydrogenase was determined according to the method of Kato et al. (24).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. D86560.

RESULTS

Isolation of a gene for CPY from fission yeast and nucleotide sequence analysis. CPY is a serine carboxypeptidase, and the carboxypeptidases have several highly conserved regions, including a serine-active residue. We attempted to isolate a gene for a CPY homolog from *S. pombe* by PCR using primers based on the three conserved amino acid sequences. The primers designated on the basis of these sequences were synthesized, and PCR was performed with the *S. pombe* cDNA library as a template. A single DNA fragment of 600 bp was amplified. The sequencing of 20 individual clones revealed that all were identical and contained an open reading frame that showed strong homology to the genes for *S. cerevisiae* CPY and other serine carboxypeptidases. With the cloned PCR fragment as a probe, a genomic library of *S. pombe* λEMBL3-*Sau*3A was screened by plaque hybridization. λEMBL3 DNA isolated from a candidate clone of about 10 kb was fragmented by restriction enzymes. These fragments were ligated into pBluescript vector and sequenced.

The nucleotide sequence of the genomic clone contained a single large open reading frame of 2,850 bp encoding a protein of 950 amino acids with one potential N-glycosylation site. The calculated M_r of the deduced peptide is 108,336. We designated this gene *cpy1*$^+$. We compared the putative polypeptide encoded by the *cpy1*$^+$ gene with the known sequences of carboxypeptidases from other sources. The C-terminal mature region that contains the catalytic region is highly conserved in other serine carboxypeptidases. The protein shows 54.4% identity to the CPY from *S. cerevisiae* (60), 57.3% identity to CPY from *Candida albicans* (35), 56.9% identity to CPY from *Pichia pastoris* (37), 50.9% identity to YBR1015 (a putative CPY homolog from *S. cerevisiae*) (4), and 30.9% identity to human protective protein/cathepsin A (14) (Fig. 1). Human protective protein/cathepsin A is a glycoprotein that associates with lysosomal β-galactosidase and neuraminidase and is deficient in autosomal recessive disorder galactosialidosis (8). The primary structure of human protective protein is highly homologous to

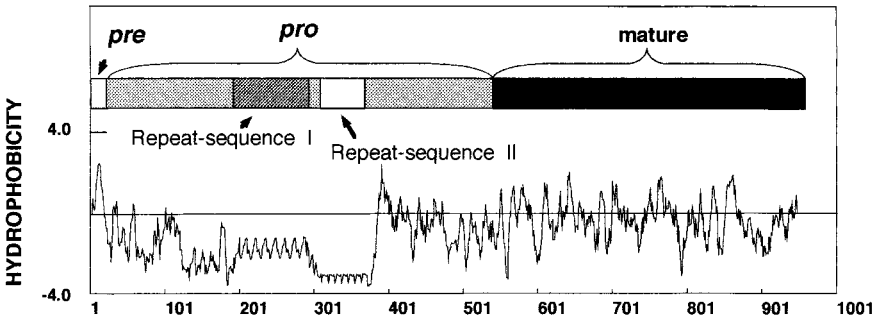


FIG. 2. Hydropathy analysis of Cpy1p. The predicted amino acid sequence of the *cpy1*$^+$ gene product was subjected to a hydropathy analysis by the method of Kyte and Doolittle (29). A scanning window of 8 amino acids was used. Hydrophobic values lie above the horizontal axis; hydrophilic values lie below. The amino acids are numbered along the horizontal axis.

those of yeast and plant serine carboxypeptidases (14). In contrast, the N-terminal pre region containing the signal sequence and the pro region containing the vacuolar sorting signal in the CPY from *S. cerevisiae* have fewer identical residues. On the basis of von Heijine's signal sequence cleavage site theory (64), the putative pre peptide of Cpy1p was calculated to extend from methionine at position 1 to alanine at position 18. In *S. cerevisiae*, the processing of pro-CPY depends on proteinase A and proteinase B. The processing site for proteinases A and B shows high similarity to the CPY of other yeasts. It is predicted that in the vacuole, the precursor protein of Cpy1p undergoes processing between valine at position 521 and lysine at position 522 by proteinase B-like protease. The pro region contains two unusual repeating sequences, repeating sequences I and II, consisting of seven contiguous repeating segments of 13 amino acids each and seven contiguous repeating segments of 9 amino acids each, respectively. The hydropathy analysis revealed that the N-terminal pro region (residues 19 to 371) containing repeating sequences I and II is very hydrophilic (Fig. 2).

Chromosomal disruption of *cpy1*$^+$. To examine the phenotypic consequences of a null allele of *cpy1*$^+$, we performed a gene deletion-disruption of this locus. A linear fragment of the *cpy1*$^+$ gene in which the 330-bp *Eco*RI-*Xba*I internal fragment was deleted and replaced with *S. pombe ura4*$^+$ gene (Fig. 3A) was used to transform haploid strain TP4-1D. Consistent with the observation that the *PRC1* gene is not essential in *S. cerevisiae*, many candidate *ura4*$^+$-disrupted *cpy1*$^+$ transformants were obtained. Several transformants were isolated, and the structure of the disrupted allele was verified by Southern blot analysis. We then examined the growth phenotype and cellular morphology of the strain in which *cpy1*$^+$ was disrupted, MTD-2. Colonies of wild-type and *cpy1*Δ cells were streaked onto yeast extract-peptide-dextrose plates and incubated at 26 and 36°C for 4 days. Both wild-type and *cpy1*Δ *S. pombe* cells grew well at 26 and 36°C; *cpy1*Δ cells did not exhibit a temperature-sensitive growth defect.

We began the characterization of carboxypeptidase activities in wild-type and *cpy1*Δ strains. Carboxypeptidase activity was measured in cell extracts from wild-type and *cpy1*Δ cells with CBZ-Phe-Leu as the substrate. Significant carboxypeptidase activity was detected in the wild-type strain, and more than 90% of total activity was decreased in *cpy1*Δ cells (Fig. 3B). We also examined the carboxypeptidase activity found in extracts of cells where *cpy1*$^+$ is expressed, using the pAL-KS$^+$ plasmid with the 4.1-kb *Sca*I-*Xba*I fragment containing the open reading frame of the *cpy1*$^+$ gene (strain MTD-2/pMT1). Therefore, disrupting *cpy1*$^+$ in these cells eliminated their capacity to synthesize carboxypeptidase, suggesting that *cpy1*$^+$ gene encodes an active carboxypeptidase.

Characterization of the *cpy1*$^+$ gene product. (i) *S. pombe* Cpy1p is a heterodimer serine carboxypeptidase. Using the specific antibody for *trpE-cpy1* fusion protein, we detected the *cpy1*$^+$ gene product. Under reducing conditions, an immunoreactive band was detected with a molecular mass of 32 kDa in *S. pombe* wild-type cell extract analyzed by SDS-PAGE (Fig. 4,

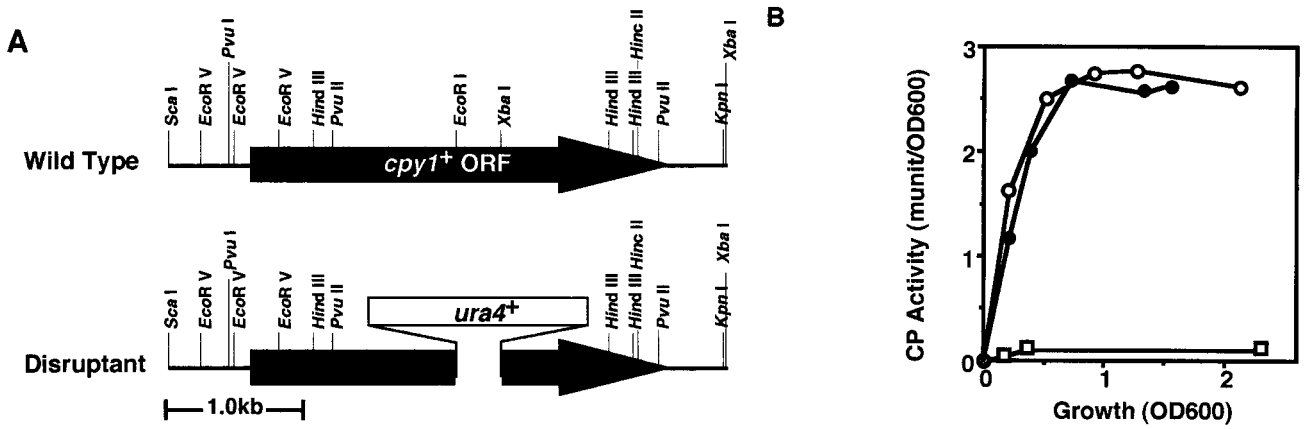


FIG. 3. Disruption of the *cpy1*$^+$ gene. (A) Restriction map and gene disruption of the *cpy1*$^+$ locus. (B) Comparison of carboxypeptidase (CP) activities of TP4-1D (wild type) (○), MTD-2 (*cpy1*Δ) (□), and MTD-2 carrying plasmid pMT1 (●) for growth phase.

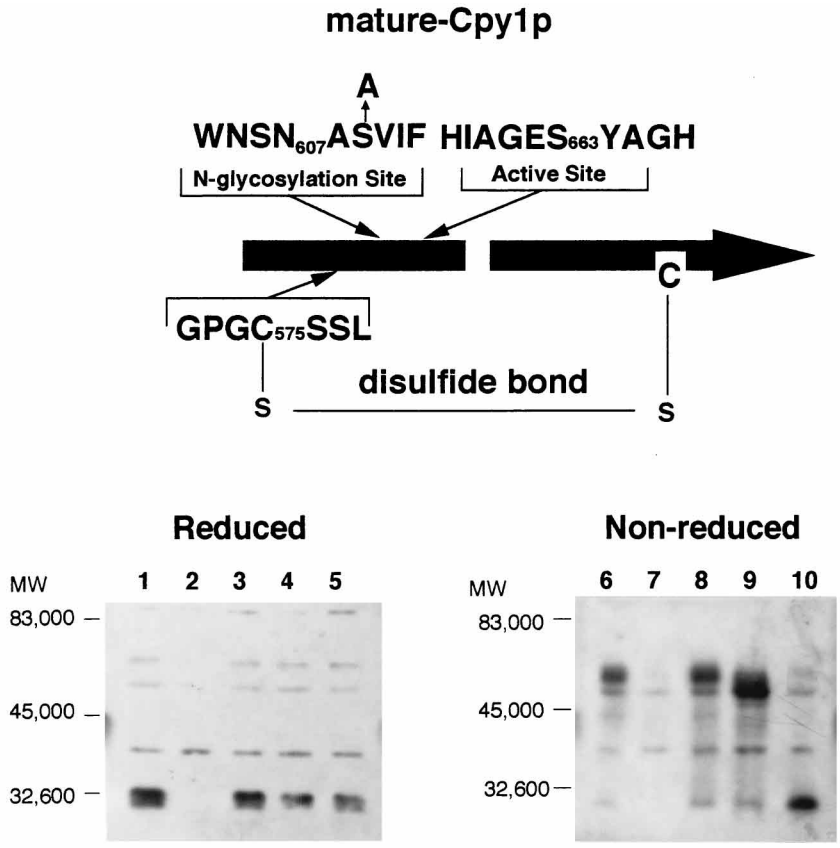


FIG. 4. Mutational analysis of Cpy1p. Western blot analysis of TP4-1D (wild-type) and MTD-2 (*cpy1*Δ) cells expressing mutant Cpy1p (S609A and C575T). Lysates of cells were subjected to electrophoresis on 10% polyacrylamide gels containing SDS. Proteins were blotted onto an Immobilon-P transfer membrane and probed with rabbit antiserum against the *trpE-cpy1* fusion protein. The molecular weight (MW) marker used was Rainbow marker (Amersham). Lanes 1 and 6, protein extracts from *S. pombe* wild-type strain TP4-1D; lanes 2 and 7, *cpy1*Δ strain MTD-2; lanes 3 and 8, MTD-2 carrying plasmid pMT1; lanes 4 and 9, mutant Cpy1p (S609A); lanes 5 and 10, mutant Cpy1p (C575T).

lane 1). In *S. pombe cpy1*Δ extract, no immunoreactive band could be detected at 32 kDa (Fig. 4, lane 2). The predicted molecular mass of the mature-Cpy1p is 48 kDa, much larger than 32 kDa. Serine carboxypeptidases from various sources have been characterized. Soluble serine carboxypeptidases which are homologous to *S. cerevisiae* CPY are divided into two classes. One is a single-chain monomer enzyme like CPY from *S. cerevisiae* and *P. pastoris*. The other is a heterodimer enzyme which consists of two polypeptide chains linked by disulfide bonds and includes serine carboxypeptidase II from wheat (6) and human protective protein/cathepsin A (14). Under nonreducing conditions, the Cpy1p shifted to approximately 55 kDa (Fig. 4, lane 6). This result indicates that *S. pombe* Cpy1p is a heterodimer serine carboxypeptidase, and the rabbit antiserum for *trpE-cpy1* fusion protein used specifically recognizes one of the two polypeptide chains.

Eleven cysteine residues are present within the predicted amino acid sequence of the deduced mature form of the Cpy1p. As these cysteine residues are highly conserved in CPY from *S. cerevisiae*, formation of disulfide bridges at the same positions are predicted in the *S. pombe* Cpy1p (13). We constructed a plasmid, pTPM-7, which carries the mutant Cpy1p (C575T). By analogy to *S. cerevisiae* CPY, Cys^{575}, predicted to form a disulfide bridge with Cys^{828}, was replaced with Thr by site-directed mutagenesis. The mutant Cpy1p (C575T), when expressed in *cpy1*Δ strain MTD-3 under both reducing and nonreducing conditions, had a molecular mass of 32 kDa (Fig. 4, lanes 5 and 10) and only 36% of the original CPY activity (Table 2). These results suggested that the two polypeptides of Cpy1p are held by disulfide bridges and function as a heterodimer.

(ii) *S. pombe* Cpy1p does not require the Asn-linked oligosaccharide for vacuolar delivery. Unlike lysosomal proteins in many mammalian cell types, which require a mannose-6-phosphate determinant for sorting, CPY from *S. cerevisiae* does not require Asn-linked oligosaccharides for vacuolar delivery (49, 57). In *S. cerevisiae* CPY, protein sorting information is contained within its pro sequence. It is not quite understood how vacuolar proteins are sorted from proteins that are destined for the cell surface in *S. pombe*. We investigated whether the

TABLE 2. Characterization of enzymatic activities of various mutant Cpy1s[a]

Strain description	Carboxypeptidase activity (mU/OD_{600})	Relative activity (%)
Wild type	2.7	100
*cpy1*Δ	<0.2	<10
*cpy1*Δ pAU-*cpy1*$^+$	2.8	104
*cpy1*Δ pAU-S609A	3.0	110
*cpy1*Δ pAU-C575T	1.0	36

[a] Cells were grown in MM to an OD_{600} of 1.0. Carboxypeptidase activities were measured as described by Stevens et al. (58) (see Materials and Methods).

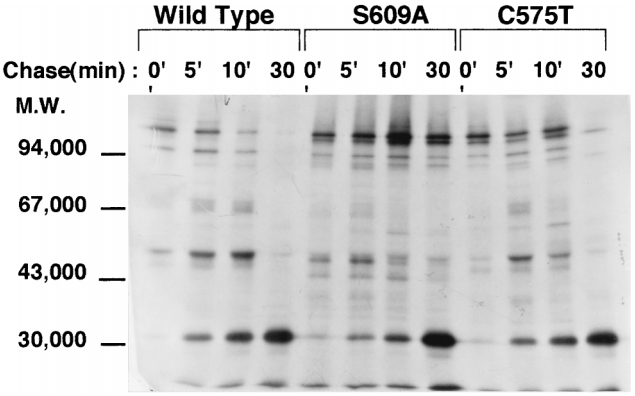


FIG. 5. Processing of Cpy1p in vivo. Cells of TP4-1D (wild type) and MTD-2 (*cpy1*Δ) expressing mutant Cpy1p (S609A and C575T) were pulse-labeled with Express-^{35}S-label for 10 min at 30°C and chased. Crude extracts of the cells and immunoprecipitates were prepared as described in Materials and Methods. The immunoprecipitates were separated on an SDS–10% polyacrylamide gel. The autoradiograms of the fixed dried gels are shown. M.W., molecular weight.

Asn-linked oligosaccharide of the *S. pombe* Cpy1p functions as the determinant for sorting to the vacuole. The predicted polypeptide of *S. pombe* Cpy1p contains only one potential acceptor site for Asn-linked glycosylation (amino acid 607). We constructed another plasmid, pTPM-6, which carries the mutant Cpy1p (S609A), in which the serine residue in the Asn^{607}-Ala-Ser sequence was substituted by alanine by site-directed mutagenesis. The mutant Cpy1p (S609A), when expressed in *cpy1*Δ MTD-3, was reduced to 52 kDa under nonreducing conditions (Fig. 4, lane 9), and it had 110% of the original carboxypeptidase activity (Table 2). Under reducing conditions, it had a molecular mass of 32 kDa (Fig. 4, lane 4), the same as that for wild-type Cpy1p. Therefore, the rabbit antiserum used probably recognizes the unglycosylated polypeptide chain. These results suggest that the mutant Cpy1p (S609A) is correctly delivered to the vacuole, and this unglycosylated Cpy1p is processed by proteinases in the vacuole. Therefore, unlike the mammalian lysosomal sorting system, the Asn-linked oligosaccharide is not the determinant for sorting to the vacuole in *S. pombe*. We believe that the determinant for sorting to the vacuole in *S. pombe* is contained within the pro sequence, as is the case with *S. cerevisiae* CPY.

Processing of the *cpy1*$^+$ gene product in vivo. In *S. cerevisiae*, the posttranslational modifications that occur on CPY during transit through early secretory organelles to the vacuole result in electrophoretic mobility differences. These differences can be seen with pulse-chase radiolabeling analysis and are indicative of CPY's compartmental location. To investigate the intracellular transport of the *cpy1*$^+$ gene product in *S. pombe*, pulse-chase analysis was performed. Wild-type and *S. pombe cpy1*Δ cells expressing the mutant Cpy1p (S609A and C575T) were pulse-labeled with 100 μCi of Expres^{35}S-label for 10 min at 30°C and were then chased for intervals of 0 to 30 min with excess unlabeled cysteine and methionine. Metabolically labeled Cpy1p was then immunoprecipitated from extracts and analyzed by SDS-PAGE followed by autoradiography.

In wild-type cells, during a 10-min pulse with Expres^{35}S-label, an immunoreactive band with an apparent molecular mass of 110 kDa was detected (Fig. 5). The predicted molecular mass of pro-Cpy1p is 106,245; thus, this 110-kDa band appears to be pro-Cpy1p. After 5 min of chase, the molecular mass of this 110-kDa form was converted to 51 kDa before finally being processed to 32 kDa. By 30 min of chase, both the 110- and 51-kDa forms had been completely processed to the 32-kDa form. On Western blot analysis, Cpy1p with a molecular mass of 32 kDa was detected under reducing conditions. Therefore, this 32-kDa form is the mature form of Cpy1p, while the 51-kDa form is a single-polypeptide intermediate Cpy1p, the pro segment of which has been removed. We found that the molecular mass of the heterodimer form of Cpy1p was approximately 55 kDa on Western blot analysis under nonreducing conditions, but this was 4 kDa larger than the kinetic intermediate that had not yet undergone processing. This difference is because Cpy1p is not denatured under nonreducing conditions. On pulse-chase analysis, Cpy1p is completely denatured, and the calculated molecular mass is 51 kDa under reducing conditions. The intermediate form is then processed to the heterodimer, detected as the 32-kDa mature form on SDS-PAGE under reducing conditions. The 110-kDa form detected on the wild-type Cpy1p was similar in size to mutant Cpy1p (S609A). In contrast, the 51-kDa form detected on wild-type Cpy1p is reduced to 48 kDa. This 48-kDa form was processed to the 32-kDa mature form as with wild-type Cpy1p. Although wild-type Cpy1p was completely processed to the 32-kDa mature form, in mutant Cpy1 (S609A), the 110-kDa pro-Cpy1p still remained after 30 min of chase. In the mutant Cpy1p (C575T), the pro-precursor form is newly synthesized (110 kDa), while in the 51-kDa intermediate form, the pro segment has been removed. The intermediate form is eventually converted to a heterodimer, which is detected as a 32-kDa mature protein on SDS-PAGE under reducing conditions, as is the case with wild-type Cpy1p.

Cpy1p does not process to the mature form in *vps34*Δ cells. In *S. cerevisiae*, genetic selection studies have identified numerous mutants that exhibit defects in vacuolar protein sorting. Analysis of these *vps* mutants has revealed a number of the *VPS* genes required for protein delivery to the vacuole. The *VPS34* gene, which encodes a phosphatidylinositol 3-kinase, is required for protein delivery to the vacuole (48, 55, 56). Recently, the *vps34*$^+$ gene, the *S. cerevisiae VPS34* gene homolog, was cloned in *S. pombe* (59). Strains in which *vps34*$^+$ is deleted are devoid of phosphatidylinositol 3-kinase activity and display temperature-sensitive growth. Mutant cells of such strains contain enlarged vacuoles. So far, there is no evidence that Vps34p is required for protein delivery to the vacuole in *S. pombe*.

To investigate Vps34p function in *S. pombe*, we performed pulse-chase analysis of Cpy1p in *vps34*Δ strain cells. After 30 min of chase, the 110-kDa form of Cpy1p was still detectable; however, the 51- and 32-kDa forms were not (Fig. 6). This indicates that Vps34p/phosphatidylinositol 3-kinase is required for protein delivery to the vacuole in *S. pombe*.

Propeptides of Cpy1p function as the vacuolar targeting signal in *S. pombe*. We have confirmed that the Asn-linked oligosaccharide of *S. pombe* Cpy1p is not required for protein delivery to the vacuole, unlike in the mammalian lysosomal delivery system. Therefore, we considered the possibility that the vacuolar sorting signal of *S. pombe* Cpy1 is contained within its pro sequence. In the case of *S. cerevisiae* CPY, the vacuolar sorting signal has been defined and shown to reside in a short stretch of amino acids at the N terminus of its pro segment QRPL (60). The similarity between the pro sequences of *S. cerevisiae* CPY and *S. pombe* Cpy1 is low, and no QRPL-like sequence is present in the *S. pombe* Cpy1 pro segment.

To identify the vacuolar sorting signal for *S. pombe* Cpy1, we have constructed a set of five *cpy1*$^+$-*SUC2* gene fusions. The hyrid proteins consisting of N-terminal segments of various lengths of *S. pombe* Cpy1 were fused in frame to the secreted enzyme *S. cerevisiae* invertase. These constructed plasmids (designated pCpy1^{N8}-Inv, pCpy1^{N23}-Inv, pCpy1^{N194}-Inv, pCpy1^{N376}-Inv,

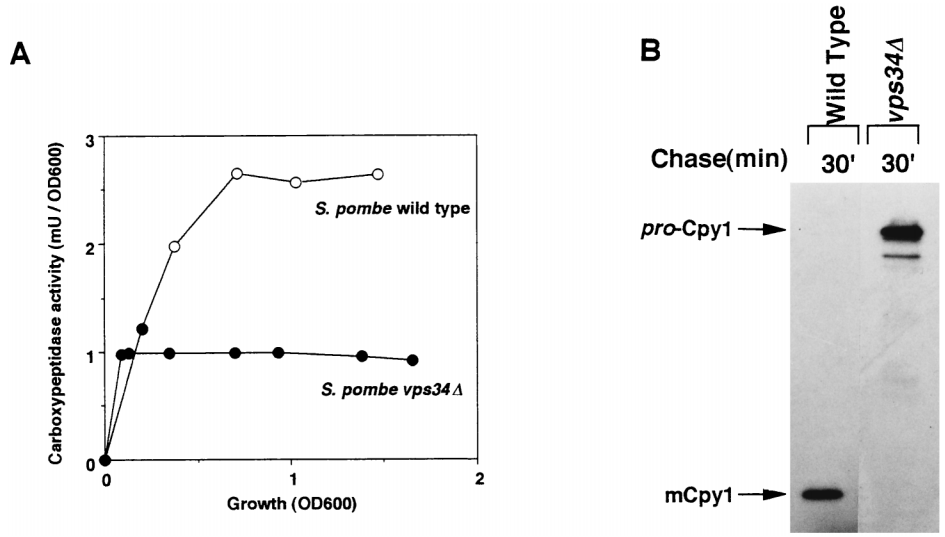


FIG. 6. Cpy1p sorting phenotype of *S. pombe* (*vps34*Δ). (A) Comparison of carboxypeptidase activities of TP4-1D (wild type) and YOD1 (*vps34*Δ). (B) Cells of TP4-1D and YOD1 were pulse-labeled with Express-^{35}S-label for 10 min at 30°C and chased for 30 min. The positions of pro-Cpy1 (110 kDa) and mature Cpy1 (mCpy1; 32 kDa) are indicated.

pCpy1^{N478}-Inv, and pCpy1^{N905}-Inv) were transformed into an *S. pombe inv1*Δ strain, NOD1. The invertase activity exhibited by the Cpy1-Inv hybrid proteins provides a useful biochemical marker with which to assess their cellular distribution (21). An invertase assay with intact cells and detergent-permeabilized cells provided a means for quantitating the amount of secreted invertase activity.

S. pombe strains harboring plasmids encoding the hybrid proteins expressed similar levels of invertase activity (data not shown). When intact NOD1 cells expressing Cpy1^{N8}-Inv, which contains only portions of the putative Cpy1 signal peptide, were analyzed for external Cpy1-Inv activity, little invertase activity was detected at the cell surface. In NOD1 cells expressing Cpy1^{N23}-Inv, which contains the entire signal peptide, 72% of total invertase activity was detected at the cell surface (Fig. 7). When the longer N-terminal portions of Cpy1p were fused to invertase, the invertase activity at the cell surface was reduced. Hybrid proteins containing 478 amino acids or more of Cpy1p fused to invertase were retained efficiently within the cell.

To determine where the Cpy1-Inv hybrid proteins are localized within the cells, we performed subcellular fractionation experiments on Ficoll density gradients. Three different enzyme were assayed in both the crude cell extract and the vacuole fraction: CPY (vacuole marker), invertase (hybrid protein), and glucose-6-phosphate dehydrogenase (cytosolic marker). The recovery of these enzyme activities in the vacuole fraction of strains expressing Cpy1^{N8}-Inv and Cpy1^{N478}-Inv is shown in Fig. 8. Invertase activity expressed in cells harboring pCpy1^{N8}-Inv did not cofractionate with the carboxypeptidase activity, demonstrating that even though Cpy1^{N8}-Inv is retained entirely within the cell, it is not being delivered to the vacuole. The hybrid protein that contains 478 amino acids fused to invertase is delivered to the vacuole, as indicated by the cofractionation with the carboxypeptidase activity (Fig. 8). These data indicated that N-terminal 478 amino acids of Cpy1p contain the vacuolar sorting signal.

Figure 7 also shows the results of the invertase plate assays. NOD1 plasmids carrying these fusion genes were patched onto an MM-fructose plate. After incubation at 30°C for 72 h, the plates were overlaid with an invertase assay solution containing 1.2% agar (40). Invertase activity can be easily detected by colorimetric assays designed to quantitate the release of glucose from sucrose hydrolysis (2, 42). Because cells do not transport sucrose across the plasma membrane, this enzyme assay can detect only the invertase activity that has been secreted from the cells. The density of the color reflects the amount of invertase activity secreted. Cells carrying plasmid pCpy1^{N8}-Inv were not stained by this assay; all of the invertase activity was internal. In contrast, cells carrying plasmid pCpy1^{N23}-Inv were found to express external invertase activity by the plate assay. The cells carrying the hybrid proteins containing 478 amino acids or more of Cpy1p fused to invertase remained. These results were in good agreement with those of the invertase liquid assay. Therefore, we suggested that the 478-amino-acid segment of Cpy1p was sufficient to direct delivery of a Cpy1-Inv hybrid protein to the vacuole and that this segment contained the putative vacuolar sorting signal of *S. pombe* Cpy1p.

When a hybrid protein containing 905 amino acids of Cpy1p was expressed in JSD1, a *vps34*Δ *inv1*Δ double mutant, almost 42% of total invertase activity was detected at the cell surface. Moreover, the Cpy1-Inv hybrid protein that should be sorted to the vacuole in the wild-type strain was also missorted to the cell surface. This indicates that the Cpy1-Inv hybrid protein is transported to the vacuole via the same pathway as Cpy1p.

DISCUSSION

The propeptide of *S. pombe* Cpy1p contains two unusual repeating sequences. The pro peptide of Cpy1p, at 504 amino acids, is much larger than those of other CPY homologs (approximately 100 amino acids). The pro peptide of Cpy1p contains two unusual repeating sequences, repeating sequences I and II. Repeating sequences I and II were compared to sequences published in various databases of the National Center for Biotechnology Information by using the BLAST and FASTA Network Service. Repeating sequence I is homologous to the proline-rich protein and histidine-rich protein of various mammals. The hydropathy analysis revealed that the N-termi-

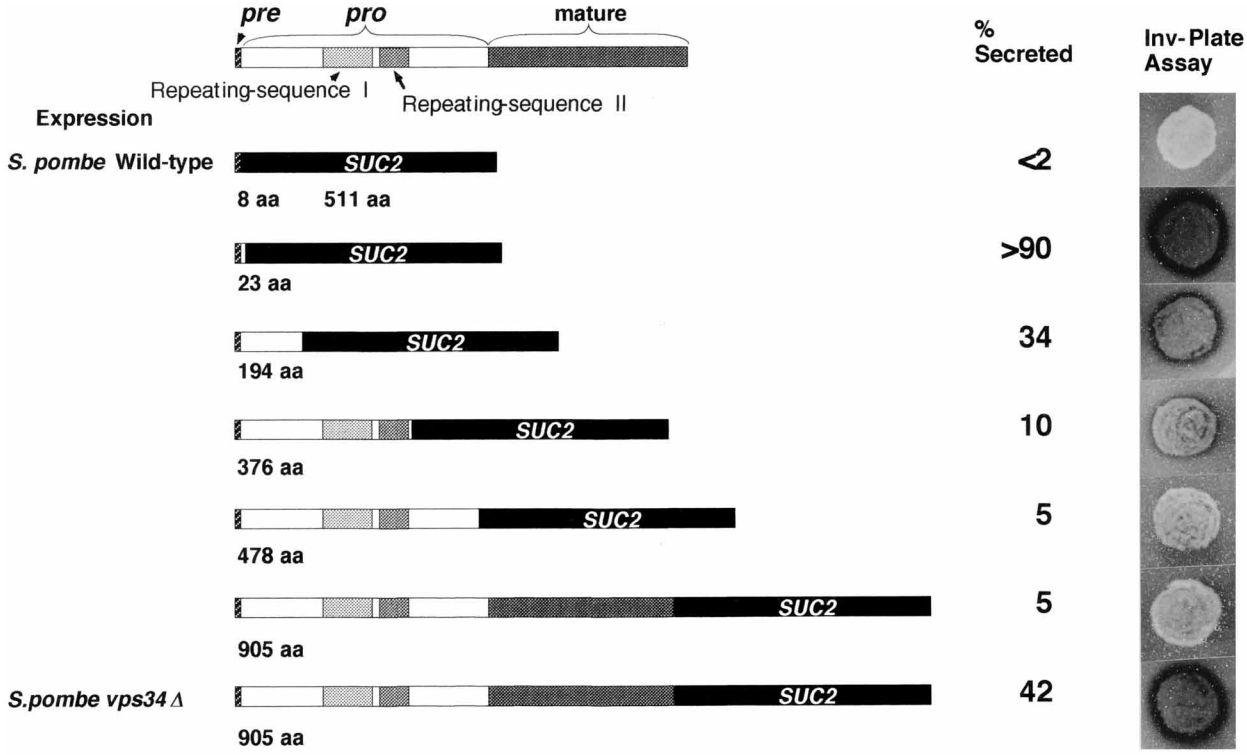


FIG. 7. Analysis of the Cpy1-Inv hybrid proteins. At the top of the figure is a schematic representation of the gene encoding Cpy1 (*cpy1*$^+$). All of the gene fusions contain the same *SUC2* gene fragment. % Secreted, amount of invertase activity secreted as determined by subtracting the background (no-plasmid control) and then calculating (extracellular/total) × 100. Where the extracellular activity was not significantly above background, the percentage secreted was considered to be <2%. (Inv-Plate assay) NOD1 carrying plasmid pCpy^{N8}-Inv, pCpy^{N23}-Inv, pCpy^{N194}-Inv, pCpy^{N376}-Inv, pCpy^{N478}-Inv, and pCpy-^{905}-Inv and JSD1 carrying plasmid pCpy1^{N905}-Inv were patched on an MM-fructose plate. After incubation at 30°C for 72 h, the plate was overlaid with an invertase assay solution containing 1.2% agar. aa, amino acids.

nal pro region containing repeating sequences I and II is very hydrophilic (Fig. 2). In several proteases, including *S. cerevisiae* CPY, the propeptide performs a chaperone-like function, aiding in the folding of the protein into a functional three-dimensional structure (20, 50, 51, 66, 69). On the basis of comparisons between the propeptide of *S. cerevisiae* CPY and heat shock proteins that actually function as chaperones, Winther and Sørensen (66) suggested that the domain of the propeptide with a relatively high frequency of charged amino acids is involved in chaperone-like activity. In *S. pombe* Cpy1p, this hydrophilic domain contained a relatively large number of charged amino acid residues (38% of this hydrophilic domain versus 22% in the mature form); therefore, these repeating sequences may be involved in chaperone-like activity.

***S. pombe* Cpy1p is a dimeric serine carboxypeptidase.** In contrast to the CPY homologs of other yeasts which are monomeric enzymes, *S. pombe* Cpy1p is a dimeric enzyme in which the 32-kDa and probably 19-kDa subunits are linked via an interchain disulfide bridge, as is the case in wheat serine carboxypeptidase II (6) and human protective protein/cathepsin A (14). The antiserum probably recognized the 32-kDa large subunit, which contained no oligosaccharide chain, indicating that the 19-kDa small subunit contained a single N-glycosylation site within the deduced *cpy1*$^+$ gene product. When *S. cerevisiae* CPY was expressed in *S. pombe*, it was transported to the vacuole and processed to the active mature form (52). This mature form was a monomeric enzyme. The possibility that the difference between the monomeric *S. cerevisiae* CPY and the dimeric *S. pombe* Cpy1p was caused by the difference in the amino acid sequences of these two enzymes was considered. A comparison between the amino acid sequence of *S. pombe* Cpy1p and those of other yeast CPYs is given in Fig. 1. A gap in the sequence of *S. pombe* Cpy1p from amino acids 680 to 692 exists. Provided that the information for the processing to the heterodimer mature form is contained

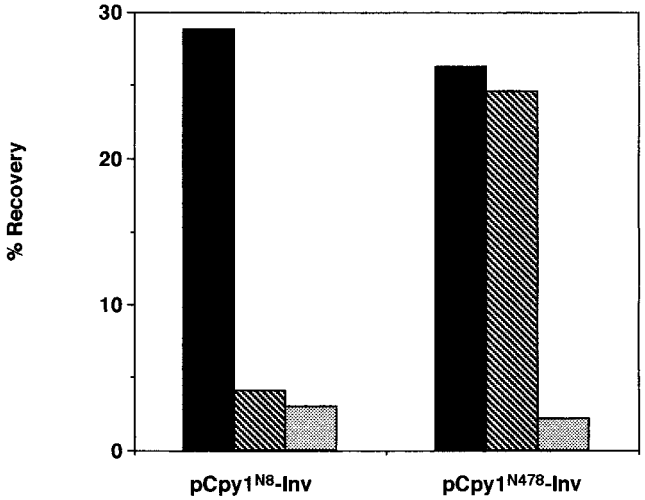


FIG. 8. Vacuole fractionation data for *S. pombe* NOD1 harboring pCpy1^{N8}-Inv and pCpy1^{N478}-Inv. The vacuole fraction was prepared as described in Materials and Methods. The percentage of recovery of each enzyme was determined by dividing the total enzyme activity recovered in the vacuole-enriched fraction by the total activity loaded on the gradient. ■, CPY; ▨, invertase; ▩, glucose-6-phosphate dehydrogenase.

in this gap, the calculated molecular mass from this gap to the C terminus end matches that of the 32-kDa subunit.

To date, all the CPY homologs characterized from yeasts consist of a single polypeptide chain. Dimeric serine carboxypeptidases have been found in higher eukaryotes such as molds (10), plants (11, 54), and mammals (14, 15). This study is the first to isolate a dimeric carboxypeptidase, *S. pombe* Cpy1p, in yeast.

The propeptide of *S. pombe* Cpy1p functions as a vacuolar sorting signal. The Asn-linked oligosaccharide of Cpy1p was not required for vacuolar protein sorting in *S. pombe*. However, unlike the original Cpy1p, the unglycosylated form had not been completely processed to maturity after 30 min of chase. Because of the delay in the correct folding in the endoplasmic reticulum, the unglycosylated pro-CPY of *S. cerevisiae* was converted to the mature form slowly in tunicamycin-treated cells (18). Winther et al. (67) reported that one of the four Asn glycosylation sites, Asn^{87}, was particularly important for the correct folding of the protein moiety. Sequence comparison made between *S. pombe* Cpy1p and *S. cerevisiae* CPY revealed that only one Asn glycosylation site of *S. pombe* Cpy1p (Asn^{609}) coincides with Asn^{87} of *S. cerevisiae* CPY, and both these sites are well conserved. We suggest that the Asn-linked oligosaccharide of *S. pombe* Cpy1p is not required for vacuolar protein sorting but may play a role in attaining the correctly folded structure.

Analysis of the Cpy1-Inv fusion protein showed that the N-terminal 478-amino-acid segment of *S. pombe* Cpy1p was sufficient to direct delivery of a Cpy1-Inv fusion protein to the vacuole in *S. pombe* and that the putative vacuolar sorting signal was contained in this segment along with two repeating motifs. The *S. pombe* Cpy1p has two repeats in the pro sequence, both of which are repeated seven times. It is interesting that the CPY receptor of *S. cerevisiae*, the *VPS10*/*PEP1* gene product, can be divided into two domains, both of which have a repeated motif that occurs seven times (19, 31, 62). Although their exact functions are unknown, these repeated motifs may be involved in the association with other proteins, e.g., the *S. pombe* CPY receptor. In *S. cerevisiae*, the N-terminal 50-amino-acid segment of *S. cerevisiae* CPY was sufficient to direct delivery of a CPY-Inv fusion protein to the vacuole (21), and the vacuolar sorting signal of *S. cerevisiae* CPY has been shown to reside in a short stretch of amino acids at the amino terminus of its prosegment QRPL (61). Additional mutants in the N-terminal region of *S. pombe* Cpy1p will be needed for further determination of the vacuolar sorting signal.

Biosynthesis and processing of *S. pombe* Cpy1p. We propose a model for the biosynthetic pathway of *S. pombe* Cpy1p (Fig. 9). When *S. pombe* Cpy1p is synthesized in its pre-pro precursor form like other known hydrolases and translocated into the endoplasmic reticulum lumen, cleavage of the signal peptide and core glycosylation take place, yielding the 110-kDa form. The core-glycosylated Cpy1p is transported to the Golgi apparatus, where Cpy1p will be modified by additional glycosylation. However, this additional glycosylation does not result in an increase of molecular mass; the Golgi form of Cpy1p is still detected as a 110-kDa protein by pulse-chase analysis. This 110-kDa form is transported to the endosome or vacuole, and its propeptide is proteolytically cleaved to give an intermediate 51-kDa form. The intermediate form is finally processed to a heterodimer which is detected in its mature form (32 kDa) by SDS-PAGE under reducing conditions. pro-Cpy1p was missorted and secreted to the cell surface in *S. pombe vps34*Δ cells. In wild-type cells, the 110-kDa form is detected after 30 min of chase, at which time it has been processed to the mature form of Cpy1p.

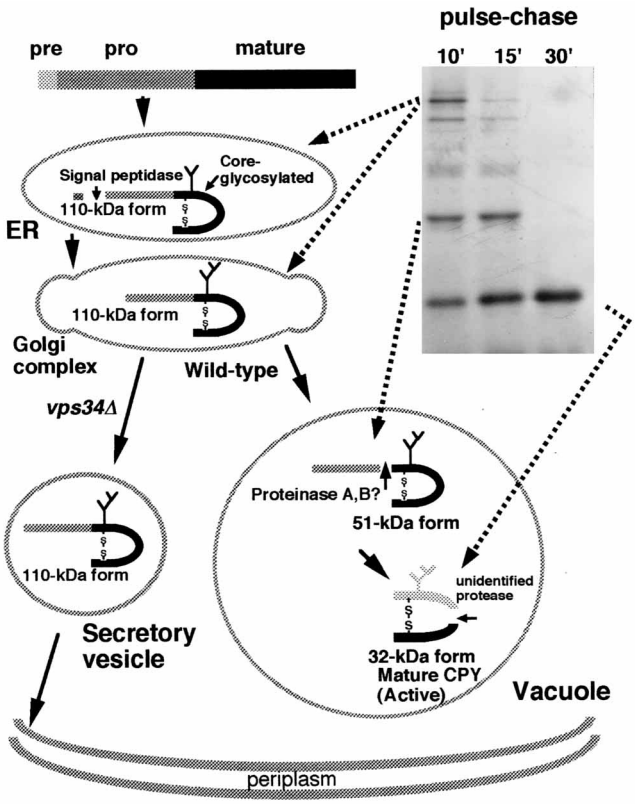


FIG. 9. A model for the biosynthetic pathway of *S. pombe* Cpy1p. See Discussion for an explanation. ER, endoplasmic reticulum.

We showed that *S. pombe* Cpy1p as a vacuolar protein marker is useful for the study of the vacuolar protein sorting mechanism in fission yeast. The $Cpy1^{N478}$-Inv fusion protein was transported to the vacuole in *S. pombe*. However, this Cpy1-Inv fusion protein was secreted in *S. pombe vps34*Δ cells. This phenotype in *S. pombe vps34*Δ cells permitted us to isolate the defective vacuolar protein sorting mutants in *S. pombe*. We have also been isolating some defective vacuolar protein sorting mutants in *S. pombe* using the Cpy1-Inv fusion protein. Characterization of these mutants will reveal not only a mechanism for the sorting of vacuolar protein in *S. pombe* but also a common protein sorting mechanism to the vacuole or lysosome conserved in all eukaryotes.

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