Gene Dosage-dependent Secretion of Yeast Vacuolar Carboxypeptidase Y

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Abstract. The structural gene for yeast vacuolar carboxypeptidase Y (*PRC1*) has been cloned by complementation of the *prc1-1* mutation. As much as an eightfold elevation in the level of carboxypeptidase Y (CPY) results when a multiple-copy plasmid containing the *PRC1* gene is introduced into yeast. Unlike the situation with a single copy of *PRC1* in which newly synthesized CPY is efficiently localized to the vacuole, plasmid-directed overproduction results in secretion of >50% of the protein as the precursor form. Secretion is blocked in a mutant that is defective at a late stage in the transport of periplasmic proteins. Unlike normal cell surface glycoproteins, secreted CPY precursor acquires no additional oligosaccharide modifications beyond those that accompany normal transport to the vacuole. In the periplasm, the CPY precursor is proteolytically activated to an enzymatically active form by an enzyme that is unrelated to the vacuolar processing enzyme. These findings suggest that proper sorting and transport of CPY is saturable. This may reflect limiting amounts of a CPY-sorting receptor, or of CPY-modifying machinery that is essential for recognition by such a receptor.

THE secretory pathway in yeast, as in more complex eukaryotic cells, is required for the transport of hydrolytic enzymes to the lysosome-like vacuole (22, 23). For example, yeast *sec* mutants blocked in transport from the endoplasmic reticulum or from the Golgi body fail to transport carboxypeptidase Y (CPY)[1] to the vacuole (29). Precursor forms of this protein accumulate at a restrictive temperature in this class of *sec* mutant cells and transport is restored when cells are returned to a permissive temperature. CPY transport to the vacuole proceeds normally in *sec* mutant cells blocked after the Golgi body step. These findings indicate that sorting of vacuolar and secretory proteins occurs at or before the Golgi body.

Unlike the targeting of lysosomal proteins in animal cells, transport of CPY to the vacuole in yeast does not require a carbohydrate determinant (26). In mammalian fibroblasts, a mannose-6-phosphate determinant directs transport of most lysosomal glycoproteins from the Golgi body to the lysosome (7, 10, 16). Nevertheless, protein structure must ultimately be responsible for targeting proteins to the lysosome. Lang et al. (18) have shown that lysosomal glycoproteins are much better substrates for the transfer of the *N*-acetyl-glucosamine-phosphate lysosomal targeting signal than similarly modified secretory glycoproteins. In order to develop a genetic means of identifying the signal(s) on CPY involved in transport of the polypeptide to the vacuole, we have cloned the CPY structural gene (*PRC1*).

In this report we show that when the *PRC1* gene is introduced into yeast on a multiple-copy 2-micron plasmid (2μ), the resulting overproduction of CPY leads to secretion of a large fraction of the newly synthesized enzyme. These results suggest that CPY transport may be mediated by a saturable component.

Materials and Methods

Strains, Growth Conditions, and Materials

A *prc1-1* strain (34) was obtained from G. Fink (Whitehead Institute, Boston, MA), and strain SEY5016α, (*sec1-1*, *leu2-3*, *leu2-112*, *ura3-52*, *gal2*) was provided by S. Emr (Caltech, Pasadena, CA). SF838-5Aα (*leu2-3*, *leu2-112*, *ura3-52*, *ade6*), SF838-1Dα (*pep4-3*, *leu2-3*, *leu2-112*, *his4-519*, *ura3-52*, *ade6*), and ISY1-7Ba (*pho80*, *leu2-3*, *leu2-112*, *ura3-52*, *his4*, *suc2*Δ*9*) were constructed by standard genetic techniques. Bacterial transformations were performed with *Escherichia coli* strain MC1061 (F$^-$*hsdR*$^-$ *hsdM*$^+$ *ara*D139Δ (*ara*ABOIC-*leu*) 7679Δ(*lac*)X74 *gal*U *gal*K *rps*L) obtained from S. Emr.

Yeast extract-peptone dextrose medium contained 1% Bacto-Yeast Extract (Difco Laboratories, Inc., Detroit, MI), 2% Bacto-Peptone (Difco Laboratories, Inc.), and 2% glucose. Wickerham's minimal medium (33) was used with the following modifications: for sulfate-free medium, chloride salts replaced all sulfate salts; for minimal medium with proline as the nitrogen source (MV-pro), 1% proline replaced ammonium sulfate and Na_2SO_4 was added to 10 mM (except in radiolabeling experiments). Supplements of leucine (30 μg/ml), uracil (20 μg/ml), histidine (20 μg/ml), and adenine (30 μg/ml) were added as needed. Unless otherwise indicated, the carbon source was 2% glucose and the yeast cultures were grown at 30°C with agitation. The absorbance of dilute cell suspensions was measured in a 1-cm cuvette at 600 nm in a spectrophotometer (model DU6; Beckman Instruments, Inc., Fullerton, CA); 1 OD_{600} unit of cells

1. *Abbreviations used in this paper:* CEN, single-copy centromere-containing plasmid; CPY, carboxypeptidase Y; E fraction, extracellular fraction; endo F, endoglycosidase F; I fraction, intracellular fraction; M fraction, medium fraction; MV-pro, minimal medium with proline as the nitrogen source; P fraction, periplasmic fraction; proCPY, CPY precursor; 2μ, multi-copy 2-micron plasmid.

corresponds to 0.15 mg dry weight. Radiolabeling experiments were initiated with exponentially growing cells at an OD_{600} of 0.5–2.

N-CBZ-L-Phenylalanyl-L-leucine, L-amino acid oxidase, horseradish peroxidase, and *o*-dianisidine were from Sigma Chemical Co. (St. Louis, MO); ^{35}S-H_2SO_4 was from ICN K&K Laboratories Inc. (Plainview, NY); IgG Sorb was from the Enzyme Center (Boston, MA); restriction endonucleases, acetylated BSA and T4 DNA ligase were from Bethesda Research Laboratories, Gaithersburg, MD; endoglycosidase F (endo F) was from Boehringer Mannheim Diagnostics Inc. (Houston, TX). Fraction II Lyticase was used in spheroplast preparation (27). Affinity-purified CPY antibody was described previously (29).

Plasmid Vectors and Recombinant DNA Methodology

Restriction endonuclease digestions and ligations with T4 DNA ligase were carried out as recommended by the suppliers. Plasmid purification, agarose gel electrophoresis, and DNA-mediated transformations of bacteria were performed by standard methods (1, 19). A yeast genomic clone bank constructed in the *LEU2* vector YEp13 (21) was used to complement the *prc1-1* mutation in cloning *PRC1*. The YEp13 plasmid containing the genomic *PRC1* insert is pTSY1. Plasmid pTSY3 was constructed by subcloning the 3.2-kb *Pvu* II-*Sal* I fragment from pTSY1 into the *Pvu* II and *Sal* I sites of the multiple-copy 2μ circle plasmid YEp24 (2μ-*PRC1*, 2) and pTSY1000 (single-copy centromere-containing plasmid (CEN)-*PRC1*) was constructed by inserting the 3.7-kb *Eco*RI-*Sal* I fragment into the *Eco*RI and *Sal* I sites in the single-copy plasmid YCp50 (17, 31) which contains the centromere region of chromosome 4 (*CEN4*). Subcloned plasmids were introduced into yeast cells by the lithium acetate transformation method (13).

A chromosomal *PRC1* deletion was constructed by transplacing (25) the genomic copy with the cloned gene into which the *LEU2* gene was inserted in place of a majority of the *PRC1* coding region. pTSY1000 was treated with *Bam*H1 followed by S1 nuclease. A 2.2-kb *Sal* I-*Xho* I fragment containing the *LEU2* gene on YEp13 was removed and treated with S1 nuclease. The two cut plasmid samples were mixed and treated with T4 DNA ligase. A plasmid conferring Amp^r Leu^+ Ura^+ was selected in a suitably marked *E. coli* strain; restriction analysis showed that the *LEU2* fragment had been inserted in the *PRC1* gene in pTSY1000. A *Hin*dIII-*Sal* I fragment containing the *LEU2* gene with *PRC1* flanking sequences was cut from this plasmid and used to transform a *PRC1 pep4-3 leu2-3 leu2-112* yeast strain. Stable Leu^+ transformants were purified and screened for immunoprecipitable CPY. One was selected that produced no detectable immunoreactive CPY ($\Delta prc1$) and this strain was used to generate yeast crude extract lacking CPY. To prepare a $\Delta prc1$ cell extract $\Delta prc1$ cells were centrifuged, resuspended to 10^{10} cells/ml in 25 mM potassium phosphate, pH 7.5, 0.2 M NaCl, followed by cell breakage in a homogenizer (Manton-Gaulin, Everett, MA). SDS was added to 1% final concentration, the extract was heated in a boiling water bath for 5 min, followed by a 15-min centrifugation in a microfuge to yield a supernatant that was used as a $\Delta prc1$ crude extract.

Enzyme Assay

Periplasmic and total CPY enzyme activities were measured by a modification of the coupled peptidase assay (35). Cells were grown to mid-log phase (OD_{600} of 1–2) in minimal medium and sedimented in a clinical centrifuge. For assay of periplasmic enzyme, cells were washed in 0.2 M potassium phosphate, pH 7.0; for assay of total activity cells were washed in 0.2 M phosphate buffer plus 1% Triton X-100. After resedimentation, cells were resuspended in 0.2 ml of 5 mM *N*-CBZ-L-phenylalanyl-L-leucine in 0.2 M potassium phosphate, pH 7.0, without (for periplasmic) or with (for total) 0.2% Triton X-100. Samples were incubated at 37°C for 30 min with gentle agitation, then heated in a boiling water bath for 3 min to stop the peptidase reaction. Released leucine was measured by the addition of 1 ml of 0.2 M potassium phosphate containing 15 μl 10 mg/ml L-amino acid oxidase, 10 μl 1 mg/ml horseradish peroxidase, 30 μl 10 mg/ml *o*-dianisidine, and 25 μl 20 mM *N*-ethyl-maleimide. After 30 min at 37°C, 1 ml of 6 N HCl was added and the sample was centrifuged for 2 min in a microfuge (Fischer Scientific Co., Pittsburgh, PA). The A_{540} of the supernatant solution was recorded: one unit is 1 μmol L-leucine released per minute and specific activity is given in milliunits per microgram dry weight.

A plate-staining procedure for CPY activity developed by E. Jones (14) was used to screen for *PRC1* transformants.

Radiolabeling, Endo F Treatment, and Immunoprecipitation

Yeast cells were grown in MV-pro medium containing 50 μM sulfate to mid-log phase, centrifuged, washed once in sulfate-free MV-pro medium and suspended in fresh MV-pro medium containing 0.5 mg/ml bovine serum albumin (BSA), unless otherwise indicated, and no added sulfate. ^{35}S-H_2SO_4 was added (100–1,000 μCi) and cells were labeled for 15 min, at which time unlabeled Na_2SO_4 was added to 10 mM final concentration. A chase period followed for either 45 or 165 min. Labeled cells were fractionated into secreted and internal fractions.

For separation of secreted from internal fractions, cells labeled in 0.5 ml of medium were sedimented in a microfuge and the supernatant medium (M) fraction was saved. The cell pellet was resuspended in 0.15 ml of 1.4 M sorbitol, 50 mM Tris-HCl (pH 7.5), 20 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 20 mM NaN_3, and 50 U lyticase/OD_{600} unit cells. Spheroplasts formed during the subsequent 45-min incubation at 30°C were sedimented in a microfuge and the supernatant (P) and pellet fractions (intracellular, I) were separated. The P fraction was adjusted to >0.5% SDS by the addition of 10 μl of 10% SDS plus 30 μl $\Delta prc1$ cell extract, and the I fraction was resuspended in 0.1 ml of 1% SDS plus 30 μl $\Delta prc1$ extract; both were heated in a boiling water bath for 3 min. Fractions were diluted to 1 ml with 12 mM potassium phosphate, pH 7.5, 0.2 M NaCl, 2% Triton X-100, followed by addition of 0.1 ml of IgG Sorb (prepared as suggested by the manufacturer). The mixture was centrifuged in a microfuge and the supernatant fraction was treated with sufficient affinity-purified CPY antibody to precipitate all the CPY antigen in 90 min at 0°C. Another aliquot of IgG Sorb was added and after 30 min at 0°C the immune complexes were sedimented and washed as before (29). Sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2% 2-mercaptoethanol, 0.01% bromphenol blue) was added and the samples were heated in boiling water for 2 min and centrifuged. Supernatant solutions were transferred to new tubes and equal aliquots were taken for SDS-gel electrophoresis as before (29). After electrophoresis gels were fixed, permeated with sodium salicylate (15) for fluorography, dried, and exposed to Kodak XAR-5 film at −80°C.

Endo F treatment (4) was carried out on immunoprecipitated samples of CPY. The washed IgG Sorb-immune complexes were heated in sample buffer at 100°C for 2 min. After removal of the IgG Sorb by centrifugation for 3 min in a microfuge the protein was precipitated from the supernatant by the addition of 4 vol of ice-cold acetone. After 30 min at −80°C the tubes were centrifuged in a microfuge for 10 min, the pellets were dried under vacuum, and then resuspended in 10 μl of 1% SDS. The tubes were heated at 100°C for 2 min and diluted to 100 μl in 50 mM potassium phosphate, pH 6.6, 50 mM EDTA, 1% Triton X-100, and 1% β-mercaptoethanol. Approximately 300 mU of endo F (4) were added and the solution incubated at 37°C for 48 h. Sample buffer was added, the tube heated in a boiling water bath for 2 min, and the samples electrophoresed as above.

Quantitation of CPY antigen was carried out by cutting out the appropriate CPY bands from the autoradiogram and eluting silver grains with NaOH (32). This procedure allowed quantitation of the relative levels of CPY synthesis in cells carrying different plasmids; these numbers were normalized for total protein synthesis (29). To determine the extent of CPY secretion, the intensity of CPY bands in the secreted (P and M) fractions was summed and compared with the total CPY synthesized (I + P + M). The percentage of secretion of CPY is thus 100 (P + M)/(I + P + M).

Results

Cloning of PRC1

Hemmings et al. (12) demonstrated that the gene identified by Wolf and Fink (34) was the structural gene for CPY (*PRC1*) by isolating strains bearing nonsense mutations at the *PRC1* locus, at least one of which produces a truncated immunoreactive fragment of the enzyme. In order to clone the *PRC1* gene, a *prc1-1 leu2-3 leu-2-112* yeast strain was transformed with a YEp13 (*LEU2*) yeast genomic bank (21) and Leu^+ transformants were selected. About 20,000 Leu^+ transformants were tested for CPY activity using the CPY plate stain (13); 16 positive colonies were detected. Quantitative assays of extracts of these transformants showed only one that contained CPY activity. Plasmid DNA was isolated from this transformant and introduced into *E. coli*. One class of plasmid was obtained (pTSY1), and a restriction map of the genomic insert in this plasmid is shown in Fig. 1. pTSY1 propagated in *E. coli* restored the wild-type Prc^+ phenotype when introduced into the *prc1* yeast strain. Enzyme activity measurements revealed an approximately fourfold increase in CPY

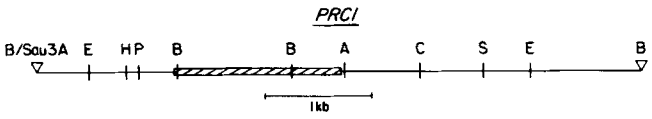

Figure 1. PRC1 insert cloned from the YEp13 yeast library. The 2.6-kb *Pvu* II-*Cla* I fragment is the smallest subclone that complements the *prc1-1* mutation. Abbreviations: A, *Acc* I; B, *Bam*HI; C, *Cla* I; E, *Eco*RI; H, *Hind*III; P, *Pvu* II; S, *Sal* I. The sequence encoding CPY is represented by the hatched box.

activity in transformants relative to wild-type untransformed cells (not shown).

The smallest subcloned region of the *PRC1* insert that complemented the *prc1-1* mutation was the 2.6-kb *Pvu* II-*Cla* I fragment. This DNA fragment actually includes the *PRC1* gene since it maps genetically to the *PRC1* locus: a strain deleted for most of the putative *PRC1* open reading frame and carrying the *LEU2* marker (see Materials and Methods) was crossed to a *prc1-1* strain, diploids were selected, sporulated, and asci dissected. In 20 asci the Prc phenotype segregated $0^+:4^-$, demonstrating that the integrated cloned gene is tightly linked to the *PRC1* (CPY structural gene) locus. In addition, the DNA sequence of the cloned *PRC1* gene confirms that it codes for the vacuolar protease CPY (Valls, L. A., and T. H. Stevens, manuscript in preparation).

Secretion of CPY is Dependent on PRC1 Gene Copy Number

To determine if any overproduced CPY escapes the normal sorting reaction in the Golgi body, cells transformed with pTSY3 or YEp24 were fractionated into I, P, and M fractions. In yeast, the cell wall retains most of the secreted proteins in the periplasmic space. These proteins can be released in a soluble form by converting cells to spheroplasts with a lytic enzyme in an osmotically supportive medium. Yeast cells were labeled with ^{35}S-H_2SO_4 for 15 min, followed by a 45-min chase period and fractionated into I and extracellular (E) fractions. Fractions corresponding to the spheroplast pellet (I), spheroplast supernatant (P), and medium (M) were immunoprecipitated with affinity-purified CPY antibody, and the resulting immunoprecipitates were solubilized and analyzed by SDS polyacrylamide gel electrophoresis and fluorography. Cells not carrying a *PRC1* plasmid have only a little extracellular CPY (Fig. 2), indicating that sorting and transport to the vacuole normally is efficient. In contrast, cells transformed with pTSY3 (multicopy *PRC1* plasmid) have a large fraction of their CPY in the E fractions as a 70-kD precursor CPY species (proCPY). The 70-kD species in the P fraction resembles the fully glycosylated intracellular proCPY (p2 CPY, 27) which is processed, probably in the vacuole, to produce the mature form of the enzyme (29). Cell lysis cannot account for the appearance of CPY in the E fractions for if this were true the E and I fractions would exhibit the same forms of CPY. Furthermore, studies with antibody specific for the soluble cytoplasmic protein phosphoglycerate kinase indicate that cytoplasmic proteins were not detected in the E fractions (P, M) when CPY was overproduced and secreted (not shown).

To define more precisely the role of gene dosage in secretion of CPY, the *PRC1* gene was subcloned into plasmids that provided varying levels of CPY overproduction. A 3.6-kb *Eco*RI-*Sal* I fragment that contained *PRC1* was subcloned into YCp50 (1) to obtain the single-copy plasmid pTSY1000 (CEN-*PRC1*). A high level overproduction plasmid was obtained by cloning the *PRC1* gene on a multiple-copy 2μ plasmid. Quantitative comparison of *PRC1* strains carrying plasmid YEp24 (no plasmid-borne *PRC1*), CEN-*PRC1*, or 2μ-*PRC1*, showed that as overproduction of CPY increased, the percentage of CPY in the E fractions increased dramatically (Table I and Fig. 2). At twofold higher-than-normal levels of CPY synthesis (pTSY1000), there is a small increase in extracellular CPY (12%) over the normal wild-type level (6%). In contrast, with six- to eightfold overproduction of CPY (2μ-*PRC1*), ~50–55% of the newly synthesized protein was directed to the cell surface. A comparable level of extracellular CPY was observed whether the nitrogen source in the growth medium was rich (ammonium chloride) or poor (proline). Thus, between two- and sixfold enhanced synthesis of CPY appears to overload the CPY-sorting apparatus. Experiments in which the *PRC1* gene was placed under the transcriptional control of the highly active *PHO5* or *ADH1* promoters did not result in levels of CPY synthesis significantly higher than were obtained with the *PRC1* promoter.

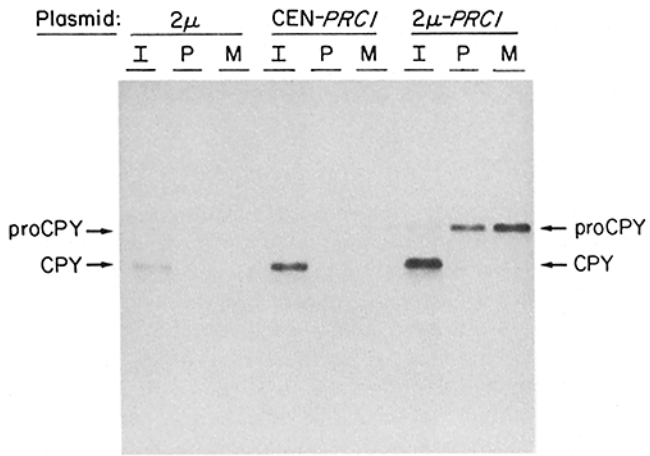

Figure 2. Secretion of CPY in a strain carrying *PRC1*-containing plasmids. Exponentially growing cultures of ISY1-7Ba (*PEP4*) carrying various plasmids (2μ:YEp24, CEN-*PRC1*:pTSY1000, 2μ-*PRC1*:pTSY3) were labeled with ^{35}S-H_2SO_4 in the presence of 0.5 mg/ml BSA for 15 min and 10 mM sulfate was added to initiate a 45-min chase. Labeled cells were centrifuged, converted to spheroplasts, and CPY immunoprecipitated from I, P, and M fractions.

Table I. Overproduction of CPY Results in Secretion of the Protein

Plasmid	Estimated *PRC1* gene copy number	Relative CPY synthesis	CPY secreted
			%
2μ	1	1	6
CEN-*PRC1*	2	2	12
2μ-*PRC1*	>5	6–8	50–55

Cultures of ISY1-7Ba carrying these plasmids (2μ:YEp24, CEN-*PRC1*:pTSY1000, 2μ-*PRC1*:pTSY3) were labeled with ^{35}S-H_2SO_4 for 15 min, chased in medium-containing excess Na_2SO_4 for 45 min, centrifuged, and fractionated into I, P, and M fractions as before. Samples were analyzed for CPY by immunoprecipitation and electrophoresis. Relative CPY synthesis was calculated by quantifying the intensity of the CPY bands (22) expressed for each strain normalized for total ^{35}S-labeled protein. The percent CPY secreted was determined by quantitation (15) of the CPY bands for the I, P, and M fractions from Fig. 2, where the percent secreted is 100 (P + M)/(I + P + M).

Secreted CPY Transits the Full Secretory Pathway

We have shown previously (29) that proCPY transits the early portion of the secretory pathway. CPY fails to be transported to the vacuole in endoplasmic reticulum-blocked and Golgi-blocked *sec* mutant cells incubated at the restrictive temperature (37°C); *sec* mutations that result in the accumulation of secretory vesicles (vesicle-blocked mutations) do not block transport of proCPY to the vacuole. If overproduced CPY transits the remainder of the secretory pathway (Golgi → secretory vesicles → periplasm), then a vesicle-blocked mutant (*sec1-1*) should not secrete proCPY at 37°C. However, if the presence of extracellular CPY is due either to cell lysis or to mechanisms independent of secretion, then the *sec1* mutation should not suppress the appearance of extracellular CPY.

Yeast strain SEY5016α (*sec1-1*) carrying plasmid pTSY3 was labeled with ^{35}S-H_2SO_4 at 25° and 37°C. Cells were converted to spheroplasts and the three fractions examined as before. At 25°C, as expected, overproduced CPY was secreted (Fig. 3). At 37°C, however, secretion of proCPY was blocked and a portion of CPY (~50%) accumulated intracellularly as proCPY, whereas *SEC1*$^+$ cells carrying plasmid pTSY3 were found to secrete a high percentage of CPY (40–50%) at 37°C (not shown). Presumably the intracellular proCPY seen in *sec1* cells at 37°C resides within secretory vesicles that accumulate in such cells (23). Thus, these results indicate that extracellular CPY must reach the periplasmic space via the secretory pathway.

Stability and Activation of Secreted proCPY

Gene dosage-dependent appearance of external CPY could be accounted for by rapid degradation of low levels of the protein secreted by cells with only one or two copies of the *PRC1* gene. This possibility was examined in a pulse-chase experiment designed to test the stability of secreted CPY. Cells were labeled for 15 min in the presence of 1 mg/ml BSA and chased for either 45 or 165 min (Fig. 4). Even in cells containing one copy of the *PRC1* gene, a small percentage of proCPY (6%) escaped proper sorting, and was stable in both the periplasm and medium fractions over a 165-min chase period (Fig. 4). The stability of CPY in the medium fraction was greatly increased when BSA was present during the labeling and chase period (not shown). Very short chase periods did not reveal any additional forms of secreted CPY. Hence, the stability of the secreted proenzyme argues strongly against saturation of extracellular CPY degradation as an explanation

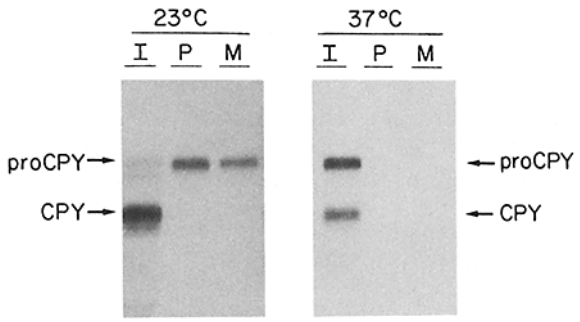

Figure 3. Secretory vesicle-accumulating mutant (*sec1-1*) blocks secretion of overproduced CPY. Yeast strain SEY5016α (*sec1-1, PEP4*) carrying plasmid pTSY3 was labeled with ^{35}S-H_2SO_4 in the presence of BSA as described in Fig. 2 at 25° and 37°C. Cells were collected, fractionated, and analyzed as before.

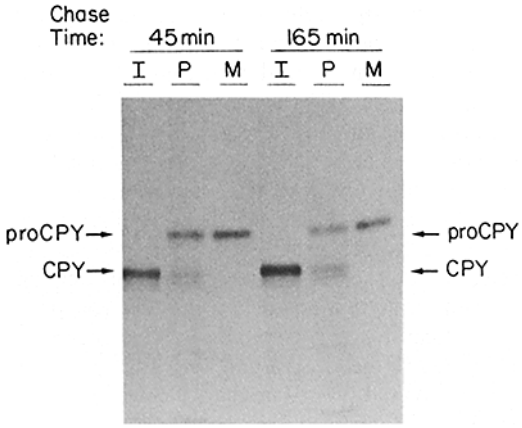

Figure 4. Low level of CPY secreted by wild-type yeast cells is stable in the extracellular fractions. Yeast strain ISY1-7Ba (*PEP4*) was labeled with BSA present as described in Fig. 2 except that one-half of the culture was chased for 165 min. Cells were collected, fractionated, and analyzed for CPY as before, except that P and M samples were adjusted to have 20-fold more material loaded on the gel than the I samples to enhance detection of secreted CPY.

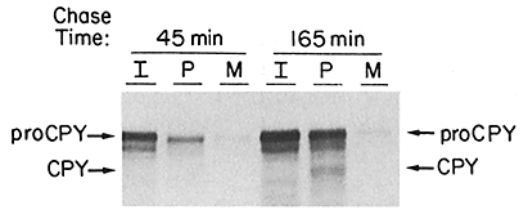

Figure 5. Secreted p2 CPY is cleaved in the periplasm. Yeast strain SF838–1Dα (*pep4-3*) carrying plasmid pTSY3 (2μ-*PRC1*) was labeled and chased as described in Fig. 4 except that BSA was not added during the labeling or chase periods. Cells were collected, fractionated, and analyzed for CPY as before.

for the gene-dosage response.

Processing of p2 CPY to mature CPY in the vacuole requires the *PEP4* gene. In the experiment shown in Fig. 4 periplasmic material with a mobility similar to mature CPY was detected after a 45-min chase and its relative abundance appeared to increase slightly during the 165-min chase. If this mature-size form is produced by a *PEP4*-dependent reaction, then periplasmic conversion should not occur in *pep4* cells. To test this possibility, *pep4* cells carrying the multiple-copy *PRC1* plasmid were ^{35}S-H_2SO_4 labeled for 15 min without BSA and chased in the presence of excess Na_2SO_4 for either 45 or 165 min. After a 45-min chase, very little 61 kD CPY was observed in the I or E fractions (Fig. 5). In contrast, after a 165-min chase period, the P fraction contained immunoreactive protein with a mobility similar to mature CPY (Fig. 5). While BSA stabilized proCPY in the medium during labeling and chase (~35% CPY in the medium in Fig. 2 vs. <5% CPY in the medium in Fig. 5), it slowed the appearance of the mature-like CPY in the periplasmic space (not shown), possibly by inhibition of some cell-surface enzyme. Extracellular conversion of proCPY thus occurs by a reaction that is independent of the *PEP4* gene, and can be inhibited by BSA in the labeling medium.

The experiment shown in Fig. 5 also demonstrated that secreted CPY (P and M fractions) had the same electrophoretic mobility as vacuolar p2 CPY, which accumulates in the

vacuole (I fraction) of *pep4* cells as a result of deficient proteolytic processing of the proenzyme (12, 29). To confirm that the polypeptide components of the secreted and vacuolar CPY precursors are similar, CPY from the I and E fractions of *pep4* cells carrying plasmid pTSY3 was treated with Endo F to remove carbohydrate (4, 29) and electrophoresed on polyacrylamide gels. Fig. 6 shows that the deglycosylated extracellular and intracellular proCPY forms comigrate, indicating that the carbohydrate on secreted proCPY is indistinguishable (by electrophoretic mobility) from that on vacuolar proCPY. This result shows that no additional detectable post-translational modifications accompany proCPY transport through the late stages of the secretory pathway.

To determine if secreted periplasmic CPY was converted to an enzymatically active form whole cells were assayed using conditions in which substrate penetrated into the periplasm, but not into the cell. *N*-CBZ-L-phenylalanyl-L-leucine, a specific substrate for CPY (35), was used to measure periplasmic activity. For comparison, cells were permeabilized with Triton X-100 to allow detection of all active CPY. Cells with a single copy of *PRC1* (Table II, strains without the *PRC1* plasmid) show background levels of periplasmic CPY activity. In contrast, *PEP4* or *pep4* cells carrying the multiple-copy *PRC1* plasmid (2μ-*PRC1*) show appreciable periplasmic CPY activity (Table II). Confirming the results in Fig. 5, which show converted CPY only in the P fraction, most of the enzymatically active CPY in the pTSY3-transformed *pep4* strain is periplasmic (Table II).

Active secreted CPY was seen in transformed cells of both mating types and in *bar1* cells carrying pTSY3 (not shown).

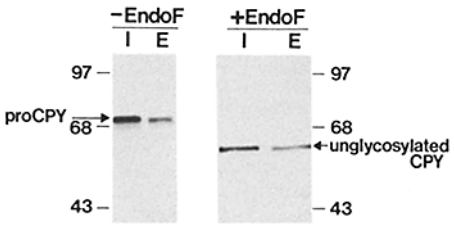

Figure 6. The molecular weights of deglycosylated secreted and vacuolar CPY presursors are similar. Yeast strain SF838-1Dα (*pep4-3*) carrying plasmid pTSY3 (2μ-*PRC1*) was labeled with ^{35}S-H_2SO_4 in the presence of BSA as described in Fig. 2. Cells were collected, fractionated, and analyzed for CPY as before except that the P and M fractions were combined into the E fraction. The I and E fractions were treated with endo F as described, and subjected to electrophoresis and autoradiography as before. Protein standards are given in kilodaltons.

Table II. Enzymatically Active CPY in the Periplasm

Strain	PEP4	Plasmid	CPY activity: Intact cells	CPY activity: Permeabilized cells
SF838				
-1Dα	−	2μ	1.2 ± 0.2	1.7 ± 0.4
-1Dα	−	2μ-*PRC1*	8 ± 1	10 ± 1
-5Aα	+	2μ	2.9 ± 0.4	36 ± 5
-5Aα	+	2μ-*PRC1*	15 ± 3	95 ± 10

Cells carrying the indicated plasmids were grown to mid-log phase, centrifuged, washed (±1% Triton X-100), and resuspended in either substrate or substrate plus 0.2% Triton X-100. CPY activity is given in milliunits per milligram dry weight. The 2μ plasmid is YEp24 and the 2μ-*PRC1* plasmid is pTSY3. The results are the average (and range) of five repeat assays.

Thus, the *BAR1* gene product, a secreted protease that may proteolytically degrade α-factor (3, 20), appears not to be responsible for activation of the CPY precursor in the periplasm.

Discussion

We have shown that a high percentage of CPY is secreted when the dosage of its structural gene (*PRC1*) is increased. Although CPY is ordinarily localized almost exclusively to the vacuole (~95%), the percentage of proenzyme that is secreted increases dramatically with elevated expression of the gene. It is possible that at some overproduction level any additional CPY synthesis results in secretion of the excess newly-synthesized protein; at six- to eightfold higher-than-normal levels of CPY production, >50% of the protein is secreted. These results are consistent with titration of some component essential for transport of CPY to the vacuole. A similar situation may explain the recent report of a mammalian cell line that overproduces and secretes a lysosomal protein in maligantly transformed but not in normal cells (6).

One interpretation of these data is that a limiting component, such as a proCPY receptor in the Golgi body, is not sufficiently abundant to handle much more than a twofold increase in CPY levels. When the receptor is saturated the unbound proenzyme is secreted. An equally plausible explanation is that some other component required for sorting, such as an enzyme that modifies CPY in a way that is essential for sorting, is limiting, and unmodified CPY is secreted. Since glycosylation is not required for sorting of CPY, oligosaccharide modification is unlikely to be the limiting reaction for proper transport (26, 29).

An alternative explanation for the gene dosage effect is that CPY overproduction results in the saturation of an extracellular protease that rapidly degrades high levels of CPY secreted by cells with new copies of *PRC1*. However, with a short labeling period designed to detect newly synthesized molecules, radiolabeled CPY is found in the E fractions even in normal cells, and this low level (6%) of secreted CPY is stable during a subsequent 165-min chase period. Hence even low levels of CPY are stable in the E fractions. These results argue strongly against limited extracellular turnover of CPY as an explanation for observing secretion at high *PRC1* gene dosage.

We have also considered the possibility that overproduction leads to aggregation of proCPY within the lumenal space of the endoplasmic reticulum or Golgi. Aggregated protein may not expose the targeting signals to the sorting component(s). However, secreted proCPY is accessible to the Golgi mannosyl transferases that act to convert p1 to p2 CPY during normal transport to the vacuole. In addition, a second yeast vacuolar enzyme, proteinase A, also exhibits overproduction-induced secretion (24), and thus proteinase A would also have to aggregate to the same extent at the same level of overproduction as CPY. Nevertheless, though we consider saturation of a limited quantity of transport sites a more likely explanation of the data, a sorting-deficient aggregate cannot be ruled out. Detection of a saturable binding component by in vitro reconstitution will allow these possibilities to be distinguished.

If a single receptor is involved in the transport of a family of vacuolar proteins, saturation by overproduction of one member should result in secretion of the related members. This remains a possibility, although in strains carrying *PRC1*

on a multicopy plasmid we have not detected secretion of enzymatically active alkaline phosphatase, or immunoreactive proteinase A polypeptide, two other vacuolar proteins (Rothman, J. H., and T. H. Stevens, unpublished observations). A more general approach may be needed to monitor secretion of a wider range of vacuolar proteins.

In the normal transit of CPY to the vacuole a 67-kD glycoprotein precursor form (produced in the endoplasmic reticulum) is glycosylated further in the Golgi body to yield a 70-kD species (p2 CPY). When CPY is secreted, the same 70-kD form is detected in the periplasm (with the same complement of carbohydrate) where it is slowly converted to an enzymatically active form. Invertase, a normally secreted enzyme, undergoes considerably more extensive glycosylation in the Golgi body where a 79–83-kD form is converted to heterogeneous forms of 100–150 kD (5). While the *sec1* experiment suggests that CPY likely transits the entire secretory pathway, the extent of glycosyl modification appears to be an inherent property of CPY itself and not simply a result of spatial separation from the glycosyl transferases that act on secreted enzymes. However, this conclusion assumes that the *sec1* block defines a unique secretory pathway and not multiple parallel pathways, a point yet to be resolved.

This situation differs from that observed in the mislocalization of lysosomal enzymes in fibroblasts from I-cell disease (mucolipidosis II) patients. Unlike secretory glycoproteins, lysosomal enzyme oligosaccharides are phosphorylated in the Golgi body. This modification allows targeting to the lysosome by interaction with a mannose-6-phosphate receptor, and prevents conversion of lysosomal oligosaccharide chains from the high-mannose to complex form (28). I-cell fibroblasts lack the enzyme that phosphorylates lysosomal enzyme oligosaccharides (11). In the absence of this modification, lysosomal enzymes undergo the normal high mannose to complex oligosaccharide conversion and are secreted (9, 28).

Conversion of the secreted proCPY to active CPY is mediated by an unknown activity; this process occurs independently of the *PEP4* gene product which is required for activation of many hydrolytic enzymes within the vacuole. Since trypsin can convert proCPY to a mature-like CPY species (8) that is enzymatically active (36), periplasmic maturation may be mediated by a secretory or plasma membrane surface endoprotease of similar specificity. The activation of the secreted precursor allows a genetic selection for mutants that mislocalize CPY. Mutations in the sorting apparatus (e.g., CPY receptor) may be obtained by selecting for cells capable of growth on a peptide which must be cleaved by periplasmic CPY to provide a necessary nutritional supplement (30). This approach should aid in the identification of molecules that participate in the sorting reaction.

In conclusion, by modulating the level of CPY synthesis through *PRC1* gene dosage experiments, we have demonstrated that proper transport of this glycoprotein to the vacuole can be overloaded. Our results suggest that CPY transport to the vacuole may be mediated by a saturable component such as a receptor. Unlike the mannose-6-phosphate receptor of mammalian cells, the yeast receptor would most likely recognize protein determinants since transport of CPY does not require glycosylation. Although excess CPY transits the entire secretory pathway, it does not undergo the glycosyl modifications characteristic of typical secreted yeast glycoproteins. This result indicates that glycosyl modifications in yeast may be dictated by structural properties inherent in the protein undergoing the modifications, and not merely by exposure to the transferases.

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