

Yeast Carboxypeptidase Y Can Be Translocated and Glycosylated without its Amino-terminal Signal Sequence

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Abstract. We have constructed a series of mutations in the signal sequence of the yeast vacuolar protein carboxypeptidase Y (CPY), and have used pulse-chase radiolabeling and immunoprecipitation to examine the in vivo effects of these mutations on the entry of the mutant CPY proteins into the secretory pathway. We find that introduction of a negatively charged residue, aspartate, into the hydrophobic core of the signal sequence has no apparent effect on signal sequence function. In contrast, internal in-frame deletions within the signal sequence cause CPY to be synthesized as unglycosylated precursors. These are slowly and inefficiently converted to glycosylated precursors that are

indistinguishable from the glycosylated forms produced from the wild-type gene. These precursors are converted to active CPY in a *PEP4*-dependent manner, indicating that they are correctly localized to the vacuole. Surprisingly, a deletion mutation that removes the entire CPY signal sequence has a similar effect: unglycosylated precursor accumulates in cells carrying this mutant gene, and >10% of it is posttranslationally glycosylated. Thus, the amino-terminal signal sequence of CPY, while important for translocation efficiency, is not absolutely required for the translocation of this protein.

SECRETORY proteins (3), many membrane proteins (2, 18), and the proteins of intracellular organelles such as the lysosome (8) are synthesized by membrane-bound ribosomes on the rough endoplasmic reticulum (ER).¹ Here the nascent proteins are transported across or inserted into the membrane. The signal hypothesis (3) is a commonly accepted model for the mechanism by which the cell directs these proteins to the ER. According to the signal hypothesis, the proteins are synthesized with hydrophobic amino-terminal extensions, 15–30 amino acids in length, known as secretory signal sequences or signal peptides. The signal sequences allow signal recognition particle to recognize the translation complexes synthesizing these proteins (45) and to mediate the binding of the polysomes to the ER membrane via the signal recognition particle receptor (10, 26). After the polysomes have bound to the membrane, the growing polypeptide chains are cotranslationally translocated across the membrane (45). During translocation, most signal sequences are removed by signal peptidase (3), and the core carbohydrate units of asparagine-linked glycosylation are added to the protein (19, 32). Prokaryotes are thought to use a similar system to direct proteins through their cell membranes (39).

The amino acid sequences of secretory signal peptides vary widely, but they have some common features. Usually

1. *Abbreviations used in this paper:* CPY, carboxypeptidase Y; ER, endoplasmic reticulum; preproCPY, unglycosylated CPY precursor with signal sequence attached; proCPY, precursor form of CPY; uproCPY, unglycosylated precursor form of CPY.

one or more basic amino acids are found near the amino terminus of the signal sequence, followed by a hydrophobic "core" region (44). The carboxy-terminal end of the signal sequence is less hydrophobic and generally contains a signal peptidase cleavage site (43). Mutational analysis has shown that point mutations and small deletions in a number of prokaryotic signal sequences can prevent or reduce translocation of the proteins (reviewed in reference 39). In contrast to the results with prokaryotes, recent studies of the yeast invertase signal sequence have shown that the yeast translocation apparatus can tolerate substantial deletion, substitution, and insertion mutations in that signal sequence (4, 16). However, if the deletions or substitutions are made large enough, translocation is prevented and the mutant invertase accumulates in the cytoplasm (16, 29). This greater sequence flexibility might be a unique property of the yeast invertase signal sequence, or it might be a general feature of eukaryotic signal sequences.

To learn more about the structural requirements for eukaryotic signal sequences we have constructed a series of mutations in the signal sequence of the yeast vacuolar enzyme, carboxypeptidase Y (CPY). CPY is a protein of the vacuole, the yeast cell's equivalent of a lysosome. It is synthesized on ER-bound ribosomes as an inactive glycosylated precursor (proCPY) (27, 40). It was previously believed that the CPY signal sequence was not removed during translocation (6). We present evidence that it is removed by signal peptidase. From the ER, proCPY is transported to the Golgi apparatus, where its carbohydrate is further elaborated, be-

fore delivery to the vacuole (40). Upon or just before arrival at the vacuole, proCPY is proteolytically cleaved to its mature, active form (6, 40). In this report we describe the effects of mutations in the CPY signal sequence. We show that these mutations decrease the efficiency of translocation of CPY across the ER membrane, and that the mutant forms of CPY appear to be posttranslationally translocated. In addition, we show that a mutant form of CPY that entirely lacks its amino-terminal signal sequence can nevertheless be translocated in vivo with >10% efficiency.

Materials and Methods

Strains, Growth Conditions, and Materials

Saccharomyces cerevisiae strain SEY2202 (MAT α *ura3-52*, *leu2-3,112*, *his4-519*) was obtained from S. Emr (California Institute of Technology, Pasadena, CA). A chromosomal deletion of the *PRC1* gene in this strain was constructed as described previously (41) to form SEY2202-*prc1*- $\Delta 2::LEU2$. EB14-11C (MAT α *ura3-52*, *leu2-3,112*, *his3*- $\Delta 200$, *lys2-801*, *prc1*- $\Delta 3::HIS3$) was constructed by standard genetic crosses. The *prc1*- $\Delta 2::LEU2$ deletion removes a large section of the coding region of the *PRC1* gene (*Bam* HI to *Bam* HI; reference 41 and Fig. 1), while the *prc1*- $\Delta 3::HIS3$ deletion removes this region and also the *Sst* II to *Bam* HI fragment, which includes the initiation codon and signal sequence coding region. A *pep4* Δ version of EB14-11C was constructed by gene transplacement (34) as previously described (1). *Escherichia coli* strain JM101 is *supE*, *thi*, Δ (*lac-proAB*), [*F'*, *traD36*, *proAB*, *lacI^q* *Z* Δ *M15*] (47). *E. coli* strain MC1061 (*F*⁻ *hsdR*⁻ *hsdM*⁺ *araD139* (*araB01C-leu*) *7679* Δ (*lac*)*X74 galU galK rpsL*) was provided by S. Emr, as was plasmid pSEY8.

Yeast cultures were grown with shaking at 30° in MV-pro liquid media (which contains 1% proline as nitrogen source) (41) supplemented with the appropriate nutrients and 50 mM potassium phosphate, pH 5.7.

Restriction enzymes, T4 DNA ligase, Klenow fragment of DNA polymerase, synthetic linkers, and DNA sequencing primers were from New England Biolabs (Beverly, MA) or Bethesda Research Laboratories (Gaithersburg, MD). Nuclease *Bal31* was from Bethesda Research Laboratories. Antibody to CPY has been described previously (40). Carrier-free ³⁵S-H₂SO₄ was from ICN (Irvine, CA). Translation grade [³⁵S]methionine was from New England Nuclear (Boston, MA). ¹²⁵I protein A was from Amersham Corp. (Arlington Heights, IL). Fraction II lyticase was prepared as described previously (37). Endoglycosidase F was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Concanavalin A Sepharose, Percoll, cycloheximide, and substrates for enzyme assays were purchased from Sigma Chemical Co. (St. Louis, MO). DE-52 cellulose was from Whatman (Maidstone, Kent, United Kingdom).

Isolation of proCPY from Culture Medium

S. cerevisiae strain EB14-11C- Δ *pep4* transformed with the multicopy *PRC1* plasmid pEBY6 was grown to a cell density of 1.5 A₆₀₀ (~1.5 × 10⁷ cells/ml) in 1 liter of MV-pro media containing 50 mM potassium phosphate, pH 6.5. During the last cell doubling, the pH was monitored and adjusted upward with 0.1 M NaOH if it fell below 5.7. The medium was clarified by centrifugation followed by filtration through a 1.2- μ m Millipore filter. It was then adjusted to pH 6.0, 0.1 μ M pepstatin, 2 mM EDTA, and

1 mM phenylmethylsulfonyl fluoride, and chilled to 4°C. The medium was mixed with 10 ml (settled volume) of DE-52 cellulose equilibrated with buffer A (20 mM Tris HCl, pH 7.0, 2 mM EDTA, 0.1 μ M pepstatin, 1 mM phenylmethylsulfonyl fluoride) and stirred at 4°C for 1 h. The resin was collected in a sintered glass funnel, washed with several volumes of buffer A, and transferred to a chromatography column. The proCPY was eluted with 400 mM NaCl in buffer A. Fractions were monitored for the presence of proCPY by SDS PAGE followed by Coomassie Blue staining and/or Western blotting. Fractions containing proCPY were pooled, concentrated using a Centricon filter concentrator (Amicon Corp., Danvers, MA), lyophilized, and stored at -20°C until use.

Amino-terminal sequence analysis of proCPY was performed by the University of Oregon Biotechnology Laboratory on a gas-phase protein sequencer (model 470A; Applied Biosystems, Foster City, CA) using Edman degradation chemistry (7).

Plasmid Constructions and Recombinant DNA Techniques

Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the suppliers. Plasmid purification, agarose gel electrophoresis, and DNA-mediated transformations of *E. coli* were performed according to standard methods (25). *E. coli* strain MC1061 was used for all plasmid manipulations and JM101 was used for M13 phage work. DNA sequencing was performed by the chain termination method of Sanger et al. (35) using the M13mpl8 and mp19 vectors (47). Plasmids were introduced into yeast cells using the lithium acetate transformation method (14).

pTSY1005 was constructed by inserting the *Cla* I to *Hind* III fragment of the *PRC1* gene (41), with an *Eco* RI linker at the *Cla* I site, into the polylinker of a single-copy *URA3* centromere plasmid (see Fig. 1 for a restriction map of *PRC1*). pEBX is the *Eco* RI to *Pvu* II *PRC1* fragment of pTSY1005 subcloned into pBR322 between *Eco* RI and *Nde* I after filling in the *Nde* I site. pEBY6 is the *Eco* RI to *Hind* III fragment of pTSY1005 subcloned into the polylinker of the multicopy (2 μ m) *URA3* vector pSEY8 (36). To construct the *prc1*- $\Delta 2$ -28 mutation, pEBX was cut with *Acc* I and *Xba* I, the ends were filled in with Klenow fragment of DNA polymerase, and *Cla* I linkers (CATCGATG) were inserted to provide an in-frame ATG adjacent to codon 28 of the *PRC1* coding region. The sequence at the deletion junction was determined by DNA sequencing and the mutation was subcloned into yeast vectors pTSY1005 and pEBY6.

Oligonucleotide-directed mutagenesis was used to introduce a glycine to aspartic acid mutation at codon 12 of the *PRC1* gene. A mutagenic primer, 5'AGTGGACAGATCTAGTCCAC3' synthesized by the triester method (13), was used to prime second-strand synthesis in M13mpl8 by the double-primer method (48). The resulting double-stranded DNA was cut with *Stu* I to destroy nonmutagenized DNA and then used to transfect *E. coli* JM101. Transfectants were screened by restriction mapping (the mutagenesis converted the *Stu* I site to a *Bgl* II site), and several with the expected restriction pattern were subjected to DNA sequence analysis. One with the desired sequence was chosen, and the mutation was subcloned into yeast vectors pTSY1005 and pEBY6.

Deletions at the unique *Stu* I site of pEBX were constructed using exonuclease *Bal31* according to the method of Maniatis et al. (25). The deleted plasmids were recircularized by ligation and amplified in *E. coli*. Then the *Eco* RI to *Bgl* II fragments from the resulting library were subcloned into the multicopy plasmid pEBY6 and again amplified in *E. coli* before being introduced into yeast.

For hydroxylamine mutagenesis, pEBX was treated with 0.4 M hydroxylamine in 0.1 M sodium phosphate at pH 6.0 for 12 h at 75°C. After ethanol

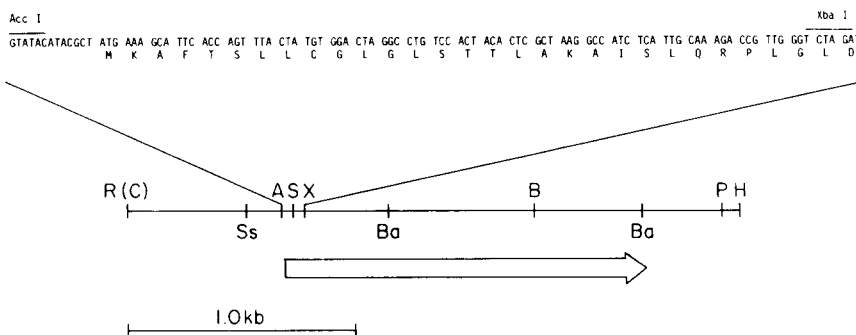


Figure 1. Restriction map of *PRC1* subclone used for transformation and mutagenesis. Open arrow indicates protein coding region. A, *Acc* I; B, *Bgl* II; Ba, *Bam* HI; C, *Cla* I; H, *Hind* III; P, *Pvu* II; R, *Eco* RI; S, *Stu* I; Ss, *Sst* II; X, *Xba* I.

Table I. CPY Activity of *PRC1* Signal Sequence Mutants

<i>PRC1</i> allele	CPY activity	
	<i>PEP4</i>	<i>pep4Δ</i>
	mU/mg	mU/mg
wild type	0.68	0.01
Asp12	0.70	0.01
Δ8-12	0.08	0.01
Δ7-12	0.03	0.01
Δ13-23	0.03	0.01
Δ9-29	0.02	0.01
Δ2-28	0.01	0.01
Δ <i>prc1</i>	0.01	0.01

EBY14-11C cells (*PEP4* or *pep4Δ*) carrying the indicated *PRC1* allele on a multicopy plasmid were broken with glass beads to prepare a lysate and assayed for CPY activity and total protein as described in Materials and Methods. CPY activity is expressed as milliunits of activity in cleaving *N*-benzoyl-L-tyrosine *p*-nitroanilide per milligram protein.

teria. The level of CPY activity in the mutant was the same as the CPY activity of wild-type transformants (Table I). In addition, pulse-chase radiolabeling experiments showed the same quantity and forms of CPY antigen present in the mutants as in transformants with wild-type plasmids (Fig. 4 A, compare wild type with Asp12), and the kinetics of conversion of precursors to mature CPY were similar for *PRC1*-Asp12 and wild-type CPY proteins (not shown).

Construction of Deletion Mutations

Since introduction of a charged amino acid had no detectable effect on signal sequence function, we turned to deletion mutagenesis to produce CPY signal sequence mutations. The observation that, when overproduced, proCPY is secreted to the cell surface and activated there by a periplasmic activity (41) allowed us to devise a screen for detecting mutants that failed to translocate proCPY. Secreted CPY activity can be detected by a filter assay in which patches of yeast cells are transferred to a filter paper disk containing a chromogenic substrate mixture. A pink spot appears on the filter if CPY activity is present at the cell surface (33). We reasoned that mutations that interfered with translocation of proCPY should reduce or prevent secretion of overproduced CPY to the cell surface, and should thus prevent the appearance of a pink spot in the filter assay.

We used nuclease *Bal31* to construct a library of mutant multicopy plasmids with deletions in the signal sequence region of the *PRC1* gene (at the *Stu I* site, see Fig. 1), as described in Materials and Methods. We then introduced the mutagenized plasmid into *S. cerevisiae* strain SEY2202-*prc1*-Δ2::LEU2, which carries a chromosomal deletion mutation

removing most of the protein-coding portion of the *PRC1* gene. Transformants were screened for CPY secretion by the filter assay. Those that gave negative filter assay results were analyzed by Western blotting, using CPY antibody as probe. This allowed us to discard transformants containing plasmids with frameshift and nonsense mutations because these made no CPY. It also enabled us to identify signal sequence mutants by the presence of uproCPY. Plasmids directing the synthesis of uproCPY were further characterized by restriction mapping. Several of them were found to contain small deletions in the region encoding the CPY signal sequence. Three deletions of various sizes (Δ8-12, Δ7-12, and Δ13-23, Fig. 3) were chosen for further study. Mutant Δ9-29 was isolated by using the same screening method on plasmids that had been mutagenized with hydroxylamine. A fifth deletion mutation (Δ2-28) was constructed by deleting the restriction fragment between *Acc I* (10 nucleotides upstream of the initiating ATG) and *Xba I* and inserting a *Cla I* linker to provide an in-frame initiation codon.

The extent of the deletion mutations was determined by DNA sequencing. The deduced amino acid sequences of the mutant regions are shown in Fig. 3. The deletions range in size from 5-27 codons and all remove at least a portion of the hydrophobic core region of the signal sequence. No new amino acids are introduced at the deletion endpoints in any of these mutations.

CPY Activity of Signal Sequence Mutants

All of the deletion mutants displayed reduced CPY activity (Table I) and reduced steady-state levels of CPY antigen relative to wild type, as detected by Western blotting (data not shown). Mutants with large deletions had lower activity than mutants with smaller deletions. When multicopy plasmids containing *PRC1* signal sequence mutations were introduced into EBY14-11C, CPY activity above background level could be detected in yeast cell lysates for all mutants except Δ2-28. The strain EBY14-11C was used for this and subsequent studies because it contains a chromosomal deletion removing the *PRC1* initiation codon and signal sequence as well as the internal coding region of the gene. Thus, in this strain, the mutant plasmids could not regain a functional signal sequence by gene conversion from the chromosome.

CPY must be proteolytically cleaved to release the active enzyme from the inactive zymogen, proCPY. This activation occurs upon or immediately before delivery of proCPY to the vacuole and requires the presence of the wild-type *PEP4* gene product (6, 40). If the active CPY produced in the signal sequence mutants also depended on the *PEP4* gene for activation, this would imply that it was correctly transported to the vacuole. To determine whether this was the case, we

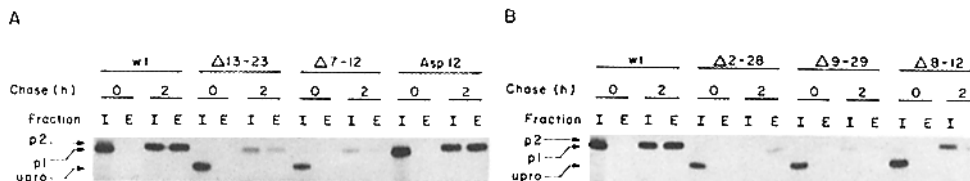


Figure 4. uproCPY chases to glycosylated proCPY in signal sequence deletion mutants. EBY14-11C-*pep4Δ* was transformed with multicopy plasmids containing the wild-type *PRC1* gene or *PRC1* signal sequence mutant genes as indicated. Cultures were pulse-la-

beled for 5 min with ³⁵S-H₂SO₄ and analyzed immediately or chased for 2 h in the presence of unlabeled Na₂SO₄, cysteine, methionine, and cycloheximide. Labeled cells were collected and converted to spheroplasts. Periplasm and culture medium were pooled to make extracellular fractions (E), spheroplasts were lysed to make intracellular fractions (I), and CPY was immunoprecipitated from both. p1, 67-kD glycosylated proCPY; p2, 69-kD glycosylated proCPY; upro, uproCPY.

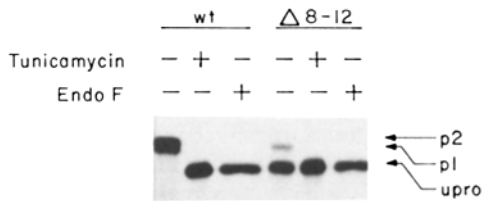


Figure 5. Effect of tunicamycin and endoglycosidase F on uproCPY. EBY14-11C-*pep4* Δ cells containing the *PRC1* gene or the *prc1*- $\Delta 8-12$ gene on multicopy plasmids were labeled for 15 min with ^{35}S - H_2SO_4 in the presence or absence of tunicamycin, as indicated. Cells were lysed by breaking with glass beads in 1% SDS and immunoprecipitated with CPY antibody as described in Materials and Methods. Immunoprecipitates of non-tunicamycin-treated cells were divided and a portion was treated with endoglycosidase F, as indicated, to remove asparagine-linked carbohydrate.

used gene transplacement (34) to create a deletion in the *PEP4* gene (1) of EBY14-11C, and transformed the resulting *pep4* Δ strain with the mutant plasmids. The *pep4* Δ transformants had no detectable internal CPY activity (Table I), indicating that the mutant forms of CPY precursor were probably using the normal pathway for CPY localization and processing.

Deletion Mutants Direct Synthesis of uproCPY

To examine the forms of CPY synthesized from the mutant plasmids, we did pulse-chase radiolabeling of the transformants, followed by immunoprecipitation and SDS PAGE. Fluorograms from such an experiment are shown in Fig. 4. In this experiment, cells were pulse-labeled with ^{35}S - H_2SO_4 for 5 min, and aliquots were taken immediately and after a 2-h chase. Internal (spheroplast) and external (periplasm plus medium) fractions were immunoprecipitated with CPY antiserum. The *PRC1* genes were present on multicopy plasmids in this experiment, so it was expected that the resulting overproduction of CPY would cause secretion of a portion of the protein (41).

When cells containing the wild-type *PRC1* gene on a multicopy plasmid are pulse-labeled under these conditions, the first CPY antigen seen is the 67-kD "p1" zymogen resulting from core-glycosylation of proCPY in the ER (Fig. 4 A, wild type time zero). The p1 is rapidly converted to the 69-kD "p2" form by carbohydrate modifications in the Golgi apparatus (40). In *PEP4* cells, the p2 form is converted to the 61-kD

mature enzyme (the only active form) before or as it arrives at the vacuole (6, 40). The experiment shown in Fig. 4 was performed using *pep4* Δ cells (which accumulate p2 in the vacuole) to improve electrophoretic resolution of the glycosylated and unglycosylated forms of CPY.

In contrast to the wild-type situation, transformants carrying signal sequence deletion mutations of *PRC1* all produced 59-kD forms of CPY during pulse-labeling. These polypeptides comigrated with uproCPY made in wild-type and mutant cells treated with tunicamycin before labeling (Fig. 5 and unpublished observation). This indicates that they represent full-length, unglycosylated proCPY. Treatment of the 59-kD polypeptides with endoglycosidase F caused no change in their mobility (Fig. 5), confirming that they contained no asparagine-linked carbohydrate. Quantitation of fluorograms from three immunoprecipitation experiments, including that shown in Fig. 4, showed that the amount of uproCPY synthesized during the pulse labeling in the mutants is equivalent to the amount of glycosylated proCPY synthesized from wild-type plasmid, within a factor of two (not shown). This indicates that the mutations do not significantly impair transcription or translation of the *PRC1* gene or substantially affect plasmid copy number. Similar results were obtained when the mutant *PRC1* genes were introduced on single copy centromere plasmids (data not shown), indicating that accumulation of uproCPY is not an artifact of overproduction.

uproCPY Chases to Glycosylated proCPY

When *PEP4* cells containing the *prc1*- $\Delta 8-12$, $\Delta 7-12$, $\Delta 13-23$, or $\Delta 9-29$ gene on a multicopy plasmid were pulse-labeled with ^{35}S - H_2SO_4 and chased in the presence of cycloheximide, CPY antigens corresponding in size to p1 and mature CPY appeared (Fig. 6 B and data not shown). These antigens, like the wild-type forms of CPY, but unlike uproCPY, could be precipitated from cell extracts with concanavalin A-linked Sepharose (Fig. 6). In addition, they were sensitive to endoglycosidase F, and did not appear when the cells were pretreated with tunicamycin (data not shown). These results indicate that these proteins contain asparagine-linked carbohydrate. Since we found that under these conditions protein synthesis (and further synthesis of CPY) was completely blocked by cycloheximide during the chase period (data not shown), the glycosylated forms of CPY must have been derived from the uproCPY that was synthesized during the pulse labeling. This indicates that glycosylation occurred posttranslationally. *PEP4* cells transformed with any of these

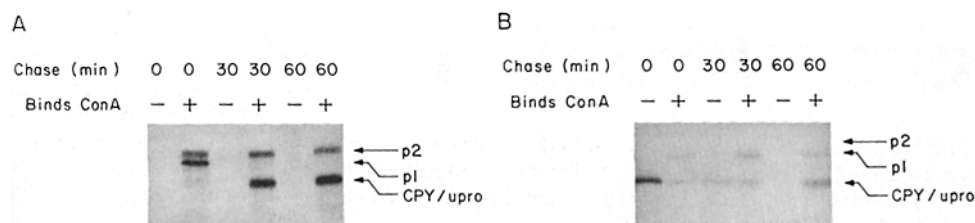


Figure 6. Concanavalin A fractionation of glycosylated and unglycosylated forms of CPY. EBY14-11C (*PEP4*) containing the (A) wild-type *PRC1* gene or (B) *prc1*- $\Delta 8-12$ on a multicopy plasmid was pulse-labeled with ^{35}S - H_2SO_4 for 15 min and chased for 0, 30, or 60 min with unlabeled Na_2SO_4 and cycloheximide. Cells were collected by centrifugation and lysed with glass beads in 1% SDS. The lysates were fractionated with concanavalin A Sepharose and the fractions immunoprecipitated with CPY antibody.

genes accumulated the p1 but not the p2 form of CPY intracellularly, whereas both p1 and p2 were seen in *pep4Δ* cells. We interpret this to mean that conversion of p1 to p2 is slow, but that p2 is converted to mature CPY in a *PEP4*-dependent manner as rapidly as it is formed. This supports our contention that these mutant forms of p2 CPY are delivered to the vacuole.

Although the mutants all made approximately equal amounts of uproCPY during a short pulse labeling, the amount of glycosylated CPY they produced after a chase varied from one mutant to another. To quantitate the fraction of uproCPY which eventually became glycosylated, we performed the pulse-chase radiolabeling experiment shown in Fig. 4 and quantitated the bands by microdensitometry. (Kinetic experiments had shown that conversion of uproCPY to glycosylated proCPY was maximal after a 2-h chase [our unpublished observation]). The results of this quantitation are presented in Table II. 45% of the uproCPY made from the *prcl-Δ8-12* gene was converted to glycosylated proCPY after a 2-h chase, whereas only 7% of the uproCPY from *prcl-Δ9-29* was glycosylated after such a chase. Intermediate levels of posttranslational glycosylation were seen for *prcl-Δ7-12* and *Δ13-23*. No uproCPY remained after a 2-h chase of cells containing the *PRCI* signal sequence deletion mutant genes. Apparently the uproCPY that does not successfully enter the secretory pathway is subject to degradation. In general, the fraction of each mutant protein undergoing glycosylation follows the same pattern as the levels of CPY activity: larger deletions lead to less glycosylation. The levels of CPY activity in the mutants, relative to wild type (Table I), appear lower than might be expected from the fraction of uproCPY that becomes glycosylated (Table II). However, the numbers are not strictly comparable. The rate of delivery of the mutant CPY proteins to the vacuole is much slower than the delivery of wild-type CPY, so that at steady state the mutants contain a substantial fraction of their CPY as uproCPY and p1. The secretion of a portion of the proCPY in these experiments is caused by overproduction of the protein from multicopy plasmids. When the *prcl-Δ8-12*, *Δ7-12*, *Δ13-23*, and *Δ9-29* genes were present on single copy centromere plasmids, >90% of the glycosylated CPY remained intracellular.

proCPY Translocated without Signal Sequence

When we constructed a deletion mutation removing the entire signal sequence of CPY along with eight adjacent amino acids ($\Delta 2-28$), we expected this mutant to make only cytoplasmic uproCPY, in analogy to the cytoplasmic form of in-

vertase made without a signal sequence (5). Remarkably, we found that even with this deletion mutation, some glycosylated proCPY was produced. Fig. 7 shows the results of a pulse-chase radiolabeling experiment with this mutant. In this experiment, culture medium, periplasm, and cell lysate were immunoprecipitated separately at various timepoints. As with the other deletion mutants, uproCPY is seen in the cells after a pulse labeling, and glycosylated forms (p1 and p2) slowly appear during a chase in the presence of cycloheximide. The p1 and p2 forms were not produced in the presence of tunicamycin. Furthermore, treatment of *prcl-Δ2-28* p1 and p2 CPY with endoglycosidase F converted them to 59-kD uproCPY (data not shown), indicating that the protein received normal ER and Golgi carbohydrate additions. Quantitation of the $\Delta 2-28$ lanes in Fig. 4B indicated that ~12% of the uproCPY synthesized in this mutant during a 5-min pulse labeling was converted to glycosylated proCPY after a 2-h chase. As with the other mutants, the remainder of the uproCPY disappeared during the chase.

In contrast to the other mutant CPY proteins, which were localized to the vacuole, $\Delta 2-28$ proCPY was secreted into the culture medium after receiving Golgi modifications to its asparagine-linked carbohydrate chains (conversion to the p2 form). Apparently none of the proCPY made in this mutant reached the vacuole, since the state of the *PEP4* gene had no effect on the forms of CPY that could be immunoprecipitated from cells carrying this mutant gene (data not shown). This explains the lack of CPY activity in cells carrying the *prcl-Δ2-28* gene. The mislocalization is not caused by overproduction, since proCPY is also efficiently localized to the cell surface in cells carrying a single copy of the *prcl-Δ2-28* gene on a centromere plasmid (not shown). Valls et al. (42) have identified mutations in the *PRCI* gene (between codons 21 and 31) that cause proCPY to be mislocalized to the cell surface. The deletion $\Delta 2-28$ extends into the region that is altered in those mislocalization mutations, providing an explanation for its effect on CPY localization. We do not yet understand why the deletion mutation $\Delta 9-29$, which removes this same region of proCPY, allows proper localization of most of that mutant protein to the vacuole. Perhaps the seven amino acids of the signal sequence that remain in this construction can somehow substitute for the normal vacuolar localization tag.

uproCPY Cofractionates with ER Membranes

In analogy to cytoplasmic invertase (5), *prcl-Δ2-28* uproCPY might be soluble in the cytoplasm because of failure to

Table II. Glycosylation of *PRCI* Signal Sequence Mutants

<i>PRCI</i> allele	Percent glycosylation
wild type	100
Asp12	100
$\Delta 8-12$	45
$\Delta 7-12$	22
$\Delta 13-23$	23
$\Delta 9-29$	7
$\Delta 2-28$	12

The gels shown in Fig. 4 were quantitated by laser densitometry. For the *prcl* mutants, percent glycosylation was calculated as glycosylated CPY after 2 h/ uproCPY after pulse $\times 100$.

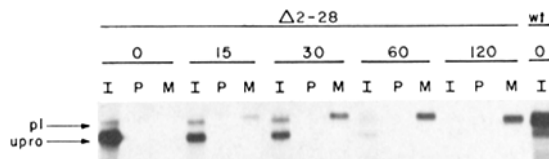


Figure 7. Glycosylated proCPY is made from a *PRCI* gene with no signal sequence. EB14-11C-*pep4Δ* transformed with a multicopy plasmid containing the *prcl-Δ2-28* gene was pulse-labeled for 15 min with $^{35}\text{S-H}_2\text{SO}_4$ and chased for the indicated times with unlabeled Na_2SO_4 , cysteine, methionine, and cycloheximide. Intracellular, periplasmic, and medium fractions (I, P, and M) were immunoprecipitated with CPY antibody. The bottom band in the right-hand lane (wild type) is glycosylated mature CPY.

associate with ER membranes. On the other hand, it might be associated with the ER membrane, and stuck at a pre-glycosylational stage of translocation. Thus the intracellular location of the uproCPY might cast some light on the nature of the defect caused by the signal sequence mutations. To address this question, we prepared a lysate of cells containing the *prcl-Δ2-28* gene on a multicopy plasmid and fractionated it by Percoll gradient centrifugation following the method of Hansen et al. for preparation of ER membranes (12). The results of this fractionation are shown in Fig. 8. uproCPY is found in the same fractions as NADPH cytochrome *c* reductase, an ER membrane marker (plasma membrane, marked by vanadate-sensitive ATPase, is also found in these fractions), and does not follow the pattern of a soluble cytoplasmic enzyme (glucose-6-phosphate dehydrogenase). This supports the idea that the uproCPY is associated with membranes. If it is membrane-associated, the membrane with which it is most likely to be associated is the ER, since a portion of the uproCPY does eventually enter the ER lumen to become glycosylated.

Discussion

The 20 amino-terminal amino acid residues of preproCPY constitute a secretory signal sequence. The primary structure of this peptide is typical of a signal sequence, and mutations within the peptide cause substantial reductions in the efficiency of translocation of proCPY. Furthermore, these first 20 amino acids have been found to support efficient translocation of invertase, when substituted for the invertase signal sequence (15). When proCPY is overproduced and secreted, its amino terminus corresponds to the expected cleavage site for signal peptidase. While we have not examined vacuolar proCPY, Johnson et al. have found that the CPY signal peptide is cleaved in the ER, in proCPY-invertase fusion proteins (15). This, in combination with our results, leads us to conclude that the signal sequence of native preproCPY is cleaved in the ER by signal peptidase.

We expected that introduction of a charged amino acid residue into the CPY signal sequence would disrupt the signal sequence function, since naturally occurring signal sequences are consistently hydrophobic (44), and prokaryotic signal sequences are functionally disrupted by mutations that introduce charged residues into them (39). Contrary to our expectations, we found that a mutation replacing glycine 12 (in the center of the CPY signal sequence) with a charged aspartic acid residue has no detectable effect on the kinetics of translocation, glycosylation, or transport of the mutant protein. This result is in contrast to the results with prokaryotes, but is similar to results seen for mutations in another eukaryotic signal sequence, that of yeast invertase. Small insertion, deletion, and substitution mutations in the invertase signal sequence have been found to have little effect on the translocation of that protein in yeast (4, 16). Furthermore, our repeated attempts to generate random point mutations in the CPY signal sequence (by thionucleotide mutagenesis; 38) that reduce or eliminate translocation have failed. Taken together, these results strongly suggest that eukaryotic signal sequences may contain redundant information for recognition by the eukaryotic cell's translocation apparatus.

We have found that deletion mutations that remove five or more amino acids of the hydrophobic core of the CPY signal

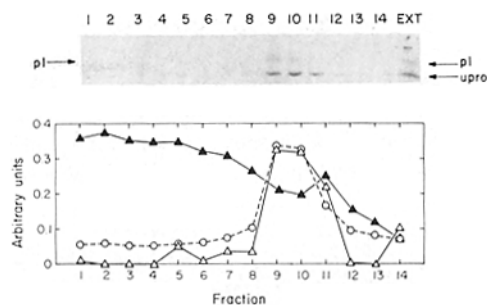


Figure 8. Percoll gradient fractionation of *prcl-Δ2-28* lysate. 1,000 A_{600} units of EBY14-11C- $\Delta pep4$ cells carrying the *prcl-Δ2-28* gene on a multicopy plasmid were used to prepare an S-10 lysate (12), which was fractionated on a Percoll density gradient (12). The gradient was collected as 1.4-ml fractions using a gradient fractionator (Haake-Buchler, Fort Lee, NJ). Fraction 1 represents the top of the gradient. (Top) 20- μ l aliquots of each fraction and of the S-10 lysate (EXT) were subjected to SDS PAGE and Western blotting, using CPY antibody and 125 I-protein A to visualize the CPY bands. (Bottom) The fractions were also assayed for NADPH cytochrome *c* reductase (O), vanadate-sensitive ATPase (Δ), and glucose-6-phosphate dehydrogenase (\blacktriangle) activities.

sequence interfere with the ability of the protein to be translocated. However, the effect of these mutations is different from that of mutations that disrupt invertase signal sequence function. Invertase mutants defective in signal sequence function produce stable cytoplasmic invertase (16). In contrast, the CPY signal sequence mutations described here lead to synthesis of unstable uproCPY, a portion of which slowly becomes glycosylated and progresses through the secretory pathway to the vacuole or the cell surface. Glycosylation of uproCPY occurs posttranslationally: it proceeds in the presence of cycloheximide, which prevents further synthesis of CPY. This may reflect a posttranslational translocation activity similar to that seen with α -factor in yeast in vitro translation systems (12, 31, 46). Alternatively, full-length uproCPY may be synthesized in a partially translocated form that slowly becomes accessible to the glycosyl transferases of the ER.

Posttranslational glycosylation has been observed for two other proteins entering the secretory pathway in yeast. Multicopy plasmid-directed overproduction of either acid phosphatase (11) or killer toxin (23) results in the accumulation of the unglycosylated fully translated preproteins in yeast. These accumulated preproteins are able to undergo posttranslational signal sequence cleavage, asparagine-linked glycosylation, and subsequent secretion in yeast (11, 23). These results gave rise to the view that accumulation of unglycosylated preprotein is a consequence of overproduction of the proteins and saturation of the ER membrane translocation machinery and/or the ER processing machinery (signal peptidase and glycosyl transferase; 11, 23). Interestingly, plasmid-directed overproduction of CPY (~1% of total yeast protein) does not saturate the ER translocation or processing machinery (41, this work). In contrast, the CPY signal sequence mutations lead to the synthesis of unglycosylated proCPY, which only slowly undergoes posttranslational translocation and/or glycosylation. Therefore, it is clear that the yeast secretory pathway is able to carry out posttranslational ER processing (signal sequence cleavage and asparagine-linked oligosaccharide addition) and possibly posttransla-

tional translocation for a number of proteins in vivo (11, 23, this work).

We have found that the CPY signal sequence can be grossly altered, and even removed entirely, without totally abolishing the ability of proCPY to enter the secretory pathway. This result appears to contradict the signal hypothesis. There are at least two possible explanations of this phenomenon that are not necessarily mutually exclusive.

First, it is possible that uproCPY is intrinsically translocatable because of some structural feature other than a signal peptide. For example, Randall and Hardy have shown that lack of tertiary structure appears to be necessary for translocation of maltose binding protein in *E. coli* (30). If lack of proper folding is similarly important in eukaryotes, uproCPY might be susceptible to translocation because of failure to fold in the cytoplasm (for instance, folding might require glycosylation or disulfide bond formation, which could only proceed in the ER lumen). This could explain why CPY signal sequence mutants behave differently than invertase signal sequence mutants. Invertase, even with a defective signal sequence attached, folds into an enzymatically active form in the cytoplasm (16) and this folding might prevent its posttranslational translocation.

Alternatively, it is possible that CPY contains an internal signal sequence that can inefficiently direct it to the ER membrane. If this is the case, such a sequence cannot readily be identified by examining the amino acid sequence of proCPY. The amino-terminal signal sequence is the most hydrophobic region of the entire preproCPY molecule (data not shown). The propeptide (amino acids 21 through 111) is highly charged (31% charged residues), and the first stretch of more than 10 uncharged amino acids begins at residue 156, well within the mature portion of the protein. It might be argued that the $\Delta 2-28$ deletion fortuitously created a signal sequence at the amino terminus of CPY, but, again, examination of the amino acid sequence fails to reveal a typical signal sequence structure. On the other hand, naturally occurring signal sequences, on which the model of a consensus signal sequence is based, may be much more uniform than is required by the cell's translocation apparatus. Kaiser et al. have found that ~20% of random in-frame open reading frames substituted in place of the invertase signal sequence could support translocation of that protein (17). We are investigating the possibility that *prc1*- $\Delta 2-28$ CPY contains a cryptic signal sequence by constructing further deletions in the *PRC1* gene to determine whether, by doing so, we can produce a form of CPY that remains totally in the cytoplasm.

We are grateful to Scott Emr for providing plasmid pSEY8 and for communicating experimental results before publication; to Tami Stenshoel for Western blotting in the early stages of mutant isolation; to Rick Feldman and Luis Valls for development of the proCPY purification protocol; to Garrick Little for oligonucleotide synthesis; to Margaret Lindorfer for amino-terminal protein sequencing; to Joel Rothman and William Hansen for helpful discussions; to Joel Rothman and Gabriele Pohl for critical readings of the manuscript; and to Elizabeth Cooksey for preparation of the manuscript.

This material is based on work supported by a National Science Foundation Graduate Fellowship (E. Blachly-Dyson) and by grants from the National Institute of General Medical Sciences (GM 32448) and the Chicago Community Trust/Searle Scholars Program.

Received for publication 16 December 1986, and in revised form 23 January 1987.

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