Yeast Carboxypeptidase Y Vacuolar Targeting Signal Is Defined by Four Propeptide Amino Acids

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Abstract. The amino-terminal propeptide of carboxypeptidase Y (CPY) is necessary and sufficient for targeting this glycoprotein to the vacuole of *Saccharomyces cerevisiae*. A 16 amino acid stretch of the propeptide was subjected to region-directed mutagenesis using randomized oligonucleotides. Mutations altering any of four contiguous amino acids, Gln-Arg-Pro-Leu, resulted in secretion of the encoded CPY precursor (proCPY), demonstrating that these residues form the core of the vacuolar targeting signal. Cells that simultaneously synthesize both wild-type and sorting-defective forms of proCPY efficiently sort and deliver only the wild-type molecule to the vacuole. These results indicate that the *PRC1* missorting mutations are cis-dominant, implying that the mutant forms of proCPY are secreted as a consequence of failing to interact with the sorting apparatus, rather than a general poisoning of the vacuolar protein targeting system.

Most proteins that function within organelles or are secreted are synthesized by a single class of cytoplasmic ribosomes. This common site of synthesis demands that proteins bound for a given subcellular compartment be sorted from other proteins and delivered to the appropriate destination. This situation also dictates that the information on a protein which specifies subcellular localization ultimately be encoded in the primary sequence of amino acids. The targeting element (Blobel, 1980) that actually mediates a sorting process may take the form of a post-translational modification to the protein or may involve the sequence of the polypeptide. Proteins whose targeting involves a number of discrete steps utilize more than one sorting signal, which acts sequentially or combinatorially to ensure proper localization of the protein (Blobel, 1980).

While the direction of protein flow from the ER to other organelles of the secretory pathway has been described (Palade, 1975; Pfeffer and Rothman, 1987), the sorting signals and cellular constituents that direct this flow are not well understood. Secretory proteins typically possess an NH₂-terminal hydrophobic signal sequence (of 18-30 amino acids) that functions as a sorting signal for ER targeting and translocation (von Heijne, 1985). The signals that direct the subsequent post-ER sorting steps are distinct from the ER signal sequence. Recent evidence has made a compelling case for a "bulk flow" model, in which proteins upon entry into the ER lumen follow the secretory pathway to the cell surface in the absence of additional targeting information (Weiland et al., 1987; Rothman, 1987). This default mechanism of secretion requires the presence of secondary, positive-acting targeting elements for retention in an intermediate compartment of the secretory pathway or for localization to the lysosome. For retention of resident ER lumenal proteins a COOH-terminal tetrapeptide sequence, Lys/His-Asp-Glu-Leu (K/HDEL), has been found to function as a secondary targeting element (Munro and Pelham, 1987; Pelham, 1988, 1989).

Targeting of proteins to the lysosome or vacuole also requires secondary signals. Many soluble lysosomal proteins in mammalian cells are phosphorylated on mannose residues, and this mannose-6-phosphate moiety serves as a lysosomal sorting signal (Sly and Fischer, 1982; Kornfeld and Mellman, 1989). It is likely that there is information somewhere within these lysosomal proteins that signals the phosphotransferase to phosphorylate this class of proteins (Lang et al., 1984); however, identification of such a discreet protein domain or element has remained elusive.

The targeting sequences on the yeast vacuolar protein carboxypeptidase Y (CPY)¹ have been extensively investigated (Rothman et al., 1989b). CPY is a glycoprotein initially synthesized as an inactive preproenzyme, which undergoes signal sequence cleavage upon ER translocation (Blachly-Dyson and Stevens, 1987; Johnson et al., 1987). The movement of the proenzyme from the ER to the Golgi apparatus can be monitored by a change from the 67-kD pl form of the ER to the 69-kD p2 form of the Golgi compartment (Stevens

¹ Abbreviations used in this paper: CPY, carboxypeptidase Y; QRPL, Gln24-Arg-Pro-Leu27.

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A postsorting proteolytic activation of proCPY to mature CPY (61 kD) takes place at or near the vacuole (Stevens et al., 1982), and this processing event is under the control of the PEP4 gene product (Hemmings et al., 1981).

The NH2-terminal region of proCPY, exposed after removal of the ER signal sequence, plays an important role in the targeting of CPY to the vacuole. A series of PRC1 deletion mutations indicated that amino acid residues 21–32 were necessary for the efficient sorting of CPY. A single point mutation resulting in missorting of proCPY was isolated after chemical mutagenesis of the entire PRC1 gene, and this lesion changed glutamine to lysine at residue 24 (Valls et al., 1987). Furthermore, a fusion protein consisting of the NH2-terminal 30 residues of preproCPY (i.e., the ER signal sequence plus 10 propeptide residues) appended to amino acid residue 3 of cytoplasmic invertase is sorted to the vacuole with ~50% efficiency, while 30 amino acids of the CPY propeptide facilitated efficient (>95%) sorting of the fusion protein (Johnson et al., 1987). Together, these results indicate that the NH2-terminal region of the CPY propeptide is both necessary and sufficient for vacuolar targeting.

To obtain a better understanding of the nature of the vacuolar sorting signal and the mechanism of protein sorting, we have carried out a more detailed mutational analysis of the CPY vacuolar targeting determinant. In this paper we describe the construction of a library of mutations in the propeptide region of CPY. This mutant analysis indicates that four contiguous residues near the NH2-terminus of the propeptide are necessary for the functioning of the CPY vacuolar localization signal. Furthermore, the PRC1 mis-sorting mutations are found to be cis-dominant. This result indicates that sorting-defective proCPY is secreted because of a failure to interact with the sorting apparatus (e.g., a CPY sorting receptor), rather than because of a general poisoning of the vacuolar protein targeting system.

**Materials and Methods**

**Strains, Growth Media, and Materials**

E. coli strains MC1061 (Casadaban and Cohen, 1980) and JM103 (Messing, 1983) were used for amplification of yeast plasmids and for DNA sequencing, respectively.

Yeast cultures were grown in minimal media containing 1% proline as sole nitrogen source (MV/pro; Stevens et al., 1982) supplemented with the appropriate nutrients. Strain HRY20-2C (MATa his3-d200 ade2-1 leu2-3,112 prc1::HIS3; Valls et al., 1987) was used for the mutant screen, CPY immunoprecipitations, and CPY enzyme assays. Strains used for the integration of PRC1 missorting alleles and carbohydrate-variant PRC1 alleles were constructed as follows. Strain SF838-9D (MATα ade6, his4-112, leu2-3,112, pep4-3, ura3-52) were constructed as follows. Strain SF838-9D (MATα ade6, his4-112, leu2-3,112, pep4-3, ura3-52) was transformed with linearized PRC1-containing yeast-integrating vectors. PRC1 alleles encoded ARG2, GUA2, and SER2, and mutations were inserted into YIp5 (Bostein et al., 1979) by cloning the 3.4-kbp Sal I-Hind III PRC1 fragment into the similarly cut vector. The result- ing plasmids, pLV16 and pLV17, were linearized at a unique Oxa NI site within the coding region of PRC1 (nucleotide 650, corresponding to amino acid residue 218) and used to transform SF838-9D strains having either wild-type PRC1 or integrated alleles of missorting mutations. Tandem integrations were obtained by selecting for Ura+ transformants and screening positives by immunoprecipitation of CPY from intracellular and extracellular fractions. Antibodies to CPY were prepared as described earlier (Stevens et al., 1982). Carrier-free [35S]H2SO4 was obtained from ICN Biochemicals, Inc. (Irvine, CA), and α-[35S]pITCP was from New England Nuclear (Boston, MA). Restriction endonucleases and other enzymes used for cloning and sequencing were from New England Biolabs (Beverly, MA), Bethesda Research Laboratories (Bethesda, MD), or Pharmacia Fine Chemicals (Indianapolis, IN). Endoglycosidase F was from Boehringer Mannheim Biochemicals (Indianapolis, IN), and substrates for enzyme assays were from Sigma Chemical Co. (St. Louis, MO). DE-52 cellulose was from Whatman Biosystems Ltd. (Maidstone, UK), IgG sorb was from the Enzyme Center (Boston, MA), and 85-mm nitrocellulose filters were from Millipore Corporation (Bedford, MA).

Region-directed mutagenesis by the method of Taylor et al. (1985a, b) was done with reagents obtained from Amersham Corp. (Arlington Heights, IL).

**Region-directed Oligonucleotide Mutagenesis**

Mutagenesis of the region of the PRC1 gene encoding the 16 amino acid region from histidine10 to leucine32 was performed as follows. A 640-bp Cla I-Bam HI fragment of the PRC1 gene was cloned into M13mp18 (cut with Hinc II and Bam HI) and into M13mp19 (cut with Eco RI and Bam HI), the latter accomplished by first making the PRC1 Cla I site blunt with dCTP, dGTP, and Klenow fragment followed by ligation of an Eco RI linker. Large scale (~80 μg) preparations of single-stranded DNA were prepared from 100-ml cultures of phage.

Two 30-nucleotide overlapping mutagenic oligonucleotides were synthesized to have a 20% mutation frequency, i.e., one in five oligonucleotides within a population would have a single base change. The oligonucleotides lacking any mutation would have the sequences 5'-AAGCCCATCTTCA-TGCGAAAGCCCTGGTGCT-GT (D051) and 5'-AGACGCTGTGGCCGTT-CATAAAGAAGCCTT-G (D052), corresponding to codons 19–28 and 25–34, respectively. To obtain a 20% mutation frequency 0.67% of any nucleotide phosphoramidite mixture was composed of "contaminating" components, i.e., 0.22% of each of three components and 99.33% of the major component. Phosphoramidites were mixed with gas-tight Hamilton syringes to avoid exposure to moisture. The oligonucleotides were purified by HPLC chromatography with tetrityl groups left on, the groups removed afterward by treatment with 3% acetic acid.

Mutagenesis according to the method of Taylor et al. (1985a, b) was initiated by annealing 50 pmol of phosphorylated primer to 10 μg of single-stranded template. Four reactions were performed in parallel using oligonucleotides D051 and D052 and the PRC1 fragment cloned into M13mp18 and mp19, giving four libraries consisting of >106 independent clones (calculated by plating a small fraction of the transfection mixture).

Four libraries, corresponding to each M13 pool, were constructed in the centromeric yeast vector pLV9 by purifying the Sst II-Apa 1 PRC1 fragment from the two M13mp18-based mutant clone pools or the Eco R I-Apa I fragment from the two mp19-based mutant pools, and inserting the fragments into an otherwise wild-type PRC1 gene (pLV9; Valls et al., 1987) from which the corresponding fragments had been excised. Each of the four libraries contained >10 000 independent clones before amplification in E. coli.

Yeast transformants were screened for extracellular CPY using the CPY colony immuno blot method (Rothman et al., 1986). Positive clones were used for preparation of plasmid DNA. The plasmids were resoluted from single E. coli transformants and reintroduced into the original yeast prc1Δ strain. Plasmids that bestowed a CPY secretion phenotype upon retransformation were screened by subclassing the mutagenized fragments into M13 vectors (Messing, 1983; Yanisch-Perron et al., 1985) and sequenced by the dideoxy chain-termination method of Sanger et al. (1977).

Actual mutation frequency was determined by preparing single-stranded DNA from 80 randomly picked phage (20 from each library). Five single-nucleotide mutations were found: GAC → TGC (Asp27 → Val); TTG → TTA (Leu34 → Phe); GCC → GCT (Ala42, silent mutation); GAC → AAC (Asp32 → Asn); TTG → TTA (Leu32 → Lys). The mutation was isolated twice, perhaps reflecting a bias in the original population of oligonucleotides, since these clones were independently isolated before amplification. Six point mutations give an approximate mutational frequency of 7.5%.

A statistical analysis for determining the completeness of the mutant li-
brary was conducted (Clarke and Carbon, 1976; Hutchinson et al., 1986). By this analysis the yeast vector libraries that were constructed and screened (>40,000 independent clones, ~60,000 screened) had >95% probability of containing a complete set of all possible single-nucleotide substitutions. It is thus highly likely that we have identified all possible single-nucleotide mutations in codons 18–34 of PRC1 that result in a CPY misrouting phenotype. A similar analysis indicates that the mutant library is not complete with respect to double mutations.

**Immunoprecipitation and Enzyme Assays**

Yeast cultures were pulse labeled in sulfate-free MV/pro +50 mM potassium phosphate, pH 5.7 with [35S]H2SO4 in the presence of 1.0 mg/ml BSA, and chased with 10 mM Na2SO4 as described previously (Valls et al., 1987). Intracellular and extracellular fractions were prepared as described previously (Stevens et al., 1986). Extracellular fractions were prepared by combining periplasmic and medium fractions before the addition of antibody. The washed immunoprecipitates were analyzed by SDS-PAGE as described previously (Stevens et al., 1986). Fluorograms were quantitated using a laser scanning densitometer (model SL-3040-XL; Biomed Instruments, Fullerton, CA). Percent secreted protein is given by the extracellular protein divided by total protein in all fractions (Intracellular + Extracellular) × 100.

Intracellular CPY activity was determined by monitoring the cleavage of benzyol tyrosine-p-nitroaniline (BTPNA) (Hemmings et al., 1981) on cells permeabilized by freeze thaw lysis in the presence of detergent. 5 A340 U of late log cultures were harvested by centrifugation, washed once with 10 mM Tris-HCl pH 8.0, 1 mM EDTA, centrifuged and resuspended in 0.7 mL of 50 mM Tris-HCl pH 7.6, 1% Triton X-100, and frozen at −80°C. Assays were initiated by the addition of 0.1 mL of 6 mM BTPNA in dimethyl formamide after thawing at 37°C. After 30 min of shaking at 37°C reactions were stopped by the addition of 10% SDS (to give a 1% final concentration) and boiling for 2 min. Assay mixtures were clarified by centrifugation and absorbance at 410 nm was measured. The specific activity of purified CPY was determined against N-[3-(2-furyl)acryloyl]-L-phenylalanyl-L-phenylalanine by measurement of the decrease in absorption at 337 nm (Winther et al., 1985).

**Results**

**Mutagenesis of the Amino-terminal Region of the CPY Propeptide**

Previous studies demonstrated that the first 30 amino acids of proCPY were necessary and sufficient as a vacuolar sorting signal (Valls et al., 1987; Johnson et al., 1987). To identify the specific amino acids critical for targeting, the region of the CPY structural gene, PRC1, encoding the sorting domain was extensively mutagenized (Fig. 1). Oligonucleotide mutagenesis was carried out with two oligonucleotides of each of the incorrect nucleotides during synthesis. This scheme should yield single mutational frequencies of ~20%, and 4% and 0.8% for double and triple mutations, respectively.

Each oligonucleotide was used to mutate a portion of the PRC1 gene cloned into M13 using the thionucleotide mutagenesis method of Taylor et al. (1985a, b). To assess the actual mutational frequency 80 randomly chosen clones were directly sequenced (Sanger et al., 1977). Six single-nucleotide mutations were identified, yielding a mutagenic frequency of 7.5%. These mutations extended from codon 20 to codon 32, indicating a wide distribution of mutations across the target sequence.

Each M13 library was used to generate a corresponding library constructed in a yeast centromere–containing vector by subcloning fragments from double-stranded M13 into a wild-type PRC1 gene (pLV9). These libraries, which contained >40,000 independent clones were amplified in E. coli and used to transform a proclΔ yeast strain. Approximately 60,000 yeast transformant colonies were screened by the colony replica-immunoblot method (Rothman et al., 1986) using anti-CPY serum. This procedure allowed the rapid identification of 200 transformants that scored positive for secretion of CPY. Colonies were picked, streaked for single colonies, clonal isolates were rescreened by the immunoblot method, and those that scored positive were used for the isolation of yeast plasmid DNA. The plasmids were amplified in E. coli and transformed into the original pcrclΔ yeast strain. Of the ~200 plasmids isolated, 50 were capable of causing a CPY secretion phenotype when reintroduced into yeast.

The mutagenized portion of the PRC1 gene from the 50 plasmids was sequenced to identify the mutations responsible for CPY secretion. 13 different lesions in the targeted region were identified. In addition to the large number of single-nucleotide lesions, two double-nucleotide mutations were obtained. The first pair gave rise to a single amino acid change Gln24 → Ser, while the second resulted in a double amino acid change, Gln24 → Glu and Pro26 → Ser. Only the single amino acid substitution mutations were investigated further. While Lys24 and Thr26 alleles were each isolated only once, most of the single-nucleotide mutations were isolated more than once, with the Ser27 allele obtained 16 times and Arg26. 6. Isolation of a large number of duplicate alleles suggests that we have conducted a nearly complete survey of mutant libraries. However, given the skewed distribution of mutations isolated (Lys24 1×, Ser27 16×) we cannot eliminate the possibility that a rare mutation could have been missed in our analysis.

**Mutational Analysis Defines a Short Domain at the Amino-terminus of proCPY**

Since the mutant screen required that the yeast colonies secrete CPY, it follows from Table I that residues 24–27, Gln24-Arg-Pro-Leu27, are required for efficient targeting of proCPY to the vacuole. CPY replica immunobLOTS are only a qualitative tool, thus, it was necessary to quantitatively determine the extent of CPY secretion for the forms of CPY encoded by the various mutant alleles. To measure the extent of CPY secretion we performed pulse-chase radiolabeling experiments on exponentially growing cultures of proclΔ cells carrying the various PRC1 missorting alleles on centromere-containing plasmids. CPY was immunoprecipitated from intracellular and extracellular fractions to assess the
Table I. Amino Acid Substitutions Leading to Missorted proCPY

<table>
<thead>
<tr>
<th>Position</th>
<th>Mutations obtained</th>
<th>Other possible single-nucleotide mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAA</td>
<td>Arg GAA CAT AAA TCA</td>
<td>CTA CCA</td>
</tr>
<tr>
<td>Gln24</td>
<td>Arg Glu His Lys Ser</td>
<td>Leu Pro</td>
</tr>
<tr>
<td>AGA</td>
<td>GGA AGC/T AAA ACA ATA</td>
<td></td>
</tr>
<tr>
<td>Arg25</td>
<td>Gly</td>
<td></td>
</tr>
<tr>
<td>CCG</td>
<td>CGG CTG ACG</td>
<td></td>
</tr>
<tr>
<td>Pro26</td>
<td>Arg Leu Thr</td>
<td></td>
</tr>
<tr>
<td>TGG</td>
<td>TCG</td>
<td></td>
</tr>
<tr>
<td>Leu27</td>
<td>Ser</td>
<td></td>
</tr>
</tbody>
</table>

Mutations that cause missorting defects in CPY. At left, 10 amino acid substitutions and their codons which were obtained by the mutant screen. The altered nucleotides are underlined. At right, other possible amino acid substitutions that could be obtained by single-nucleotide changes in the codons for the positions 24-27. Phenotypically silent mutations and stop codons would not have been found by this screen.

Table II. Summary of Phenotypes of PRC1 Point Mutants

<table>
<thead>
<tr>
<th>PRC1 allele</th>
<th>% CPY in intracellular fraction*</th>
<th>Intracellular CPY activity (% wild-type)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>Arg24</td>
<td>47</td>
<td>32</td>
</tr>
<tr>
<td>Glu24</td>
<td>31</td>
<td>35</td>
</tr>
<tr>
<td>His24</td>
<td>51</td>
<td>56</td>
</tr>
<tr>
<td>Lys24</td>
<td>31</td>
<td>21</td>
</tr>
<tr>
<td>Ser24</td>
<td>29</td>
<td>34</td>
</tr>
<tr>
<td>Gly25</td>
<td>79</td>
<td>78</td>
</tr>
<tr>
<td>Arg26</td>
<td>13</td>
<td>34</td>
</tr>
<tr>
<td>Leu26</td>
<td>87</td>
<td>64</td>
</tr>
<tr>
<td>Thr26</td>
<td>83</td>
<td>87</td>
</tr>
<tr>
<td>Ser27</td>
<td>18</td>
<td>16</td>
</tr>
</tbody>
</table>

* Average of three determinations by quantitating immunoprecipitations (Fig. 2).
† Average of two determinations using the BTPNA substrate.

The fate of newly synthesized CPY during its transit through the yeast secretory pathway. The immunoprecipitated protein was electrophoresed and subsequently detected by fluorography. As seen in Fig. 2, during a 1-h chase period CPY is secreted only weakly in certain mutants (e.g., Gly25, Leu26, Thr26), while other mutations result in a stronger phenotype (e.g., Lys24, Ser25, Arg26, Ser27). Quantitation of the data in Fig. 2 is presented in Table II. It is clear that individual mutations within the four amino acid stretch perturb this vacuolar sorting domain to various degrees.

Mutations in the CPY sorting domain result in missorting of only CPY and not other vacuolar proteins such as proteinase A (data not shown). Furthermore, cell lysis is not the cause of the appearance of CPY in the extracellular fractions, since immunoprecipitation of these fractions with antiserum directed against the cytosolic protein phosphoglycerate kinase results in <10% of this protein being found extracellularly in any mutant. The same percentage is found in extracellular fractions of wild-type cells (data not shown). This finding is supported further by the observation that mutant proteins are secreted as proCPY precursors, while intracellular CPY in each mutant is efficiently processed to the mature 61-kD species (Fig. 2). Because no mature CPY is found in extracellular fractions derived from mutant cells, it is unlikely that any CPY is released from the cell via the vacuole. The sec1 dependence of one of these point mutations, Lys24, suggests that the mutant CPYs exit the cell by traversing the late secretory pathway (Valls et al., 1987). Lastly, secretion of CPY cannot be the result of overproduction-induced mislocalization (Stevens et al., 1986; Rothman et al., 1986), since the mutants synthesize comparable amounts of CPY as wild-type cells (Fig. 2; quantitation not shown), and similar results were obtained when the missorting PRC1 alleles were first integrated into the chromosome (Valls, 1988). (PRC1 missorting alleles are designated upper case PRC1 to reflect the fact that they are cis dominant.)

CPY Encoded by PRC1 Missorting Alleles Is Enzymatically Active

The intracellular processing of CPY (Fig. 2) suggests that the mutant cells produce a proCPY species which is recognized by the vacuolar processing proteases. This contention is supported by the PEP4 dependence of this conversion (shown below). Thus, the perturbation of protein structure...
caused by prcl mutations manifests itself in a sorting-
defective phenotype while not affecting vacuolar activation
of the CPY precursor.

CPY activity assays were performed on mutant and wild-
type cells to determine whether mutant species which comi-
grate with the wild-type protein are enzymatically active.
One would predict that if fully active the amount of CPY ac-
tivity in the mutants would reflect the proportion of CPY protein found in the intracellular fractions. The assay results
(Table II) suggest that the 61-kD species in the mutants is
indeed as fully active as the wild-type protein, since the per-
cent of cell-associated CPY activity closely correlates with the proportion of CPY found in the intracellular fraction by immunoprecipitation.

To determine whether the secreted fraction of proCPY en-
coded by the PRC1 missorting alleles differs from the form of the protein correctly targeted to the vacuole, we isolated the extracellular fraction of CPY from Lys24 and Ser27 mu-
tant cultures under conditions that favor extracellular activa-
tion of proCPY (Winther, 1989). CPY from these culture supernatants was purified to homogeneity using a
CPY affinity column (Johansen et al., 1976), and assayed for CPY specific activity. The PRC1-Lys24 and PRC1-Ser27
forms of CPY had 91% and 103%, respectively, of wild-type
CPY specific activity. Thus, it is unlikely that either the in-
tracellular or extracellular forms of CPY encoded by the
PRC1 missorting alleles differ substantially in overall struc-
ture from wild-type CPY.

**PRC1 Missorting Mutations Are cis-dominant**
The sorting-defective phenotypes described above can be en-
visioned to result from the mutant forms of proCPY either failing to interact with the sorting apparatus or by generally disrupting the vacuolar protein sorting process. To deter-
mine whether secretion results from a poisoning of vacuolar
protein sorting by mutant proCPY, it was necessary to moni-
tor the sorting of wild-type CPY in the presence of the al-
tered forms. To distinguish between wild-type and mutant
forms of CPY, we employed alleles of PRC1 which produced
CPY having three rather than four sites for asparagine-linked
glycosylation (Winther, 1989). The two alleles used, encod-
ing CPY-CHOa and CPY-CHOd, result from oligonucleo-
tide-directed mutagenesis of the first and fourth glycosyla-
tion sites on CPY (the CHO site is most NH2-terminal),
respectively. The changes at these sites are Thr126 → Ala
(preventing glycosylation at Asn124) and Asn97 → Gln. The
CPY proteins carrying either amino acid change are indistin-
guishable from wild-type with respect to enzyme kinetics.
That is, the purified proteins' CPY-CHOa and CPY-CHOd
have values of kcat/Km identical to the purified wild-type
protein (Winther, 1989). Thus, the amino acid changes, and
the carbohydrate changes that result from them, appear not
to disrupt the structure of the enzyme.

To test whether carbohydrate variants of CPY are sorted
efficiently, pep4 cells carrying integrated alleles of PRC1-
CHOa and -CHOd were subjected to pulse-chase immuno-
precipitation of CPY. As can be seen in Fig. 3 A, both PRC1-
CHOa and -CHOd encode forms of proCPY that are retained
in the intracellular fraction with high efficiency. While the
experiment shown in Fig. 3 A was carried out with pep4 cells, the same efficient sorting (95% intracellular) is seen
for these carbohydrate variants in PEP4 cells, wherein
vacuolar proCPY (67 kD) is converted to the mature 59-kD form at the same rate (1s = 6 min) as wild-type proCPY
(Hasilik and Tanner, 1978). Therefore, both carbohydrate
variants of CPY behave identically to wild-type CPY with
respect to vacuolar delivery and proteolytic maturation.

When carbohydrate-variant and wild-type forms of CPY
are expressed in the same cells, both the 69-kD (wild-type)
and 67-kD (CHOa or CHOd) proCPY species are found ex-
clusively in the extracellular fraction (Fig. 3 B). This ob-
servation indicates that wild-type proCPY and carbohydrate-
variant proCPY do not affect each other’s sorting during
transit of the secretory pathway.

To determine the effect of sorting-defective CPY on the
targeting of CPY-CHOa and -CHOd to the vacuole, mutant
alleles of PRC1 were first integrated into the chromosome to
replace the endogenous wild-type allele. To obtain the same
level of expression of both carbohydrate-variant and sorting-
defective CPY in the same cell, PRC1-CHOa and -CHOd alleles were tandemly integrated adjacent to the PRC1 missort-
ing alleles as depicted in Fig. 4. This was accomplished by
choosing as a site for integration an Oxa NI restriction site
within the PRC1 gene. This site lies between carbohydrate
addition sites CHOa and CHOd. When PRC1-CHOa is di-
rected to integrate at the Oxa NI site into the chromosome
containing a missorting PRC1 allele, the duplication consists

![Figure 3](https://example.com/figure3.png)

Figure 3. CPY lacking a single asparagine-linked
Carbohydrate addition is properly sorted to the
vacuole. Cells were radiolabeled and the CPY spe-
pes immunoprecipitated and visualized as de-
scribed in Fig. 2. (A) pep4 cells (SF838-9D) har-
borating a chromosomal copy of either PRC1-CHOa
or -CHOd. (B) pep4 cells harboring a chromo-
somal tandem integration of a wild-type PRC1 gene
flanked by either PRC1-CHOa or -CHOd.
Figure 4. Generation of tandem integrants of differentially marked PRC1 genes. Cells harboring a single chromosomal allele encoding sorting-defective CPY (PRC1*) were transformed with a YIp5 integrating plasmid containing a carbohydrate-variant allele of PRC1 (-CHOa or -CHOd) after cleavage of the plasmid at an Oxa NI site (nucleotide 650 of PRC1). Integration at this site results in PRC1 alleles each bearing a single mutation (A) or a wild-type gene flanked by a PRC1 allele doubly marked by missorting and glycosylation site mutations (B).

of a PRC1 missorting allele flanked by a PRC1-CHOa allele (Fig. 4 A).

As seen in Fig. 5 A, the sorting of CPY-CHOa is not perturbed by the Arg24 mutant form of CPY, since the only extracellular CPY is the 69-kD proCPY species (Arg24), while the intracellular species is predominantly the 67-kD species (CHOa). Similarly, the secretion of CPY-Ser27 has no effect on the efficient sorting of CPY-CHOa. To be certain that the carbohydrate alteration affected neither sorting nor missorting, tandem integrations were also constructed to mark the sorting-defective PRC1 alleles. The PRC1-CHOa allele was directed to integrate at the Oxa NI site, and this resulted in a PRC1 gene carrying both sorting-defective and carbohydrate-variant mutations, flanked by a wild-type PRC1 gene (Fig. 4 B). pep4 cells harboring a chromosome of this configuration would be expected to missort and secrete a CPY molecule of 67 kD while properly sorting the 69-kD species. The immunoprecipitations presented in Fig. 5 B fully support this expectation, as both the PRC1-Glu24-CHOa and PRC1-Ser27-CHOa forms of proCPY are secreted in the same cells that effectively localize wild-type proCPY to the vacuole. Thus, the PRC1 missorting are cis-dominant, indicating that there is no interaction between various forms of proCPY during the vacuolar sorting process.

Discussion

Nature of the Vacuolar Targeting Signal

The experiments described in this paper identify the residues Gin24-Arg-Pro-Leu27 (QRPL) in the CPY propeptide as critical for localization of the protein to its resident organelle, since single mutations at any of these positions cause severe defects in the efficiency of CPY sorting to the vacuole. This set of residues presumably becomes accessible upon cleavage of the 20 amino acid signal sequence in the ER (Fig. 6). Whether

Figure 5. Sorting of coexpressed carbohydrate-variant and sorting-defective proCPY. pep4 (SF838-9D) cells harboring tandem integrations of the configurations depicted in Fig. 4 were radiolabeled, fractionated, and the CPY was immunoprecipitated and visualized as described for Fig. 2. (A) Tandem duplication of PRC1 as depicted in Fig. 4 A results in secretion of 69 kD proCPY. (B) Tandem duplication as shown in Fig. 4 B results in secretion of 67 kD proCPY.
the mature NH2-terminus of the protein) in its propeptide contains the sequence QNPL (14 amino acids NH2-terminal proteinase A sufficient for localization to the yeast vacuole. As with CPY (Johnson et al., 1987), the propeptide of proteinase A contains an element sufficient for vacuolar localization, yet there is no region in the propeptide of proteinase A resembling the QRPL CPY targeting element. Interest-

ingly, a third soluble yeast vacuolar protein, proteinase B, is efficiently sorted to the vacuole if additional random amino acid changes resulting in a decreased efficiency of sorting to the vacuole are illustrated. The four contiguous residues in bold type, Glu24-Arg-Pro-Leu27, constitute the critical residues for this vacuolar sorting domain. SP indicates the site for signal sequence cleavage.

This four amino acid domain constitutes a complete targeting element per se is less certain. While this small domain is necessary for the integrity of such an element, experiments that address its autonomous function would be required to answer the question of sufficiency. Johnson and co-workers (1987) have determined that 30 residues of the propeptide (plus the signal sequence) are sufficient to efficiently (>95%) direct the secretory protein invertase to the vacuole, while 10 propeptide residues function somewhat less efficiently (~50%). Two experimental results argue that the lower efficiency of this construct may be a manifestation of problems in context. Firstly, it has been shown that deletion of amino acid residues 29-31 of preproCPY (PRC1-ΔX3; Valls et al., 1987) results in a missorting phenotype. This deletion alters the position of the QRPL element by three residues relative to the rest of the propeptide domain. Secondly, the failure to find point mutations in residues 28-34 that affect CPY sorting suggests that the precise sequence of these residues is unimportant for this vacuolar delivery signal. The CPY-invertase fusion protein containing only 10 CPY propeptide residues (Johnson et al., 1987) might be efficiently sorted to the vacuole if additional random residues were inserted at the junction site to form a longer "spacer" region. If so, a reasonable model for the mechanism of missorting caused by deletion of residues 29-31 would be an alteration in accessibility of QRPL rather than the removal of residues critical for sorting.

While many of the PRC1 missorting mutations result in uncharged residues being substituted for charged and vice versa, such changes are not required for missorting, e.g., Glu24 → Ser, Pro26 → Leu and Leu27 → Ser strongly affect CPY sorting. These mutations do not appear to result in consistent changes in predicted secondary structure (Chou and Fasman, 1978). Whether the mutations have a local or global effect on protein structure may have to await crystallographic analysis of wild-type and mutant proCPY proteins.

Using a gene-fusion approach, Emr and colleagues (Klionski et al., 1988) have identified a targeting signal on proteinase A sufficient for localization to the yeast vacuole. As with CPY (Johnson et al., 1987), the propeptide of proteinase A contains an element sufficient for vacuolar localization, yet there is no region in the propeptide of proteinase A resembling the QRPL CPY targeting element. Interestingly, a third soluble yeast vacuolar protein, proteinase B, contains the sequence QNPL (14 amino acids NH2-terminal to the mature NH2-terminus of the protein) in its propeptide (Moehle et al., 1987); however, it is not known whether targeting information resides in the proteinase B propeptide. Thus, it is not yet clear how universal (or unique) the QRPL vacuolar targeting element will be among soluble vacuolar proteins.

**Mechanism of CPY Sorting**

The molecular details of sorting CPY from secretory proteins remain to be elucidated (Rothman et al., 1989a, b; Robinson et al., 1988). However, while no direct biochemical evidence for a receptor has yet been obtained certain aspects of this process have been recently clarified. It is known that CPY, when missorted, exits the cell via the late (sec1-dependent) secretory pathway after undergoing the glycosyl modifications typical of passage through the Golgi apparatus (Valls et al., 1987). These results argue for a late Golgi, prevacuolar bypass of the sorting mechanism in the PRC1-missorting mutants. Because the secreted form of CPY in these mutants, 69 kD glycosylated proCPY, is indistinguisable from the species which is missorted when CPY is overproduced, it seems reasonable to suggest that the step at which missorting occurs in the former is the same sorting step which is saturated in the latter.

Conventional protein sorting models invoke receptor-mediated mechanisms (e.g., mannose-6-phosphate receptor-mediated sorting of some soluble lysosomal proteins), but a high-affinity receptor may not be necessary for efficient sorting (Burgess and Kelly, 1987; Toole et al., 1989). These mechanisms of protein sorting demand the existence of a finite number of affinity steps; the greater the affinity, the fewer the number of steps that are needed. If the sorting of CPY is mediated by a series of cooperative weak interactions, such associations might include aggregation of CPY (self-association). The separation of a proCPY aggregate from other luminal contents of the Golgi would also require, at a minimum, a weak but cumulative affinity between monomers within the aggregate and a membrane component. A prediction of this model is that individual vesicles bound for the vacuole would contain a subset of vacuolar proteins rather than a complete spectrum of vacuolar proteins. The fact that mutations which disrupt CPY sorting are cis-dominant severely constrains any such aggregation or multimerization models. Mutant proCPY molecules do not interact with wild-type during the sorting process; if such an interaction existed, the proportion of mutant CPY found intracellularly would be higher than in cells synthesizing only the mutant form (i.e., mutations would be recessive), or extracellular amounts of wild-type proCPY would be higher (i.e., mutations would be dominant) or both would be observed (codominance).

The cis-dominance implies either of two mechanisms: proCPY is sorted from secretory proteins as a monomer, or, if any multimerization occurs during the sorting process, the PRC1 missorting mutations must destroy both the ability of proCPY to oligomerize and must also destroy the interaction of the monomer with the sorting apparatus. In terms of the aggregation model above, the sorting determinant must mediate both such interactions during wild-type proCPY sorting since experimental evidence strongly suggests that the small propeptide domain, identified by mutations and gene-fusion studies, is both necessary and sufficient for sorting.
proCPY. Therefore, a dual role of oligomerization and targeting for this region of the protein seems unlikely.

These models serve to illustrate the possible mechanisms of sorting of CPY. If sorting is strictly receptor mediated, the PRCI missorting mutations must disrupt the binding site structure on proCPY for the receptor, since there would be no other interaction that facilitates sorting. The evidence presented here supports this model of proCPY sorting.

Conclusions

Our studies indicate that the tetrapeptide QRQL found very near the NH₂-terminus of proCPY functions as a vacuolar targeting signal. While the context of this targeting element seems important for efficient vacuolar sorting, the precise sequence of amino acids surrounding the element (residues 21–23 and 28–34) is less important. Furthermore, wild-type and sorting-defective forms of CPY expressed in the same cell do not affect each other’s sorting (or missorting). This clear cis-dominant behavior of the PRCI missorting mutations is most consistent with a failure of the mutant forms of proCPY to interact with the sorting apparatus (i.e., CPY sorting receptor). The availability of these PRCI missorting alleles has now made possible a genetic analysis of pseudoalleles that may aid in the identification and characterization of the putative CPY sorting receptor.

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The Journal of Cell Biology, Volume 111, 1990 368

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368