The core of the vacuolar targeting signal of yeast carboxypeptidase Y (CPY) is recognized by the receptor Vps10p and consists of four contiguous amino acid residues, Gln²⁴-Arg-Pro-Leu²⁷, near the amino terminus of the propeptide (Valls, L. A., Winther, J. R., and Stevens, T. H. (1990) J. Cell Biol. 111, 361–368; Marcusson, E. G., Horazdovsky, B. F., Cereghino, J. L, Gharakhanian, E., and Emr, S. D. (1994) Cell 77, 579–586). In order to determine the sequence specificity of the interaction with the sorting receptor, substitutions were introduced into this part of the propeptide by semirandom site-directed mutagenesis. The efficiency of vacuolar sorting by the mutants was determined by immunoprecipitation of CPY from pulse-labeled cells. It was found that amino acid residues Gln²⁴ and Leu²⁷ were the most important ones. While it appears that Gln²⁴ is essential for proper function, Leu²⁷ can be exchanged with the other hydrophobic amino acid residues, isoleucine, valine, and phenylalanine. Tolerance toward various substitutions for Arg²⁶ is fairly high, while substitution of Pro²⁶ for uncharged amino acid residues also resulted in only weak missorting. In addition to the low requirement for sequence conservation, the position of the targeting element relative to the amino terminus of the propeptide was analyzed and found not to be critical.

Eukaryotic cells consist of a number of compartments, each with a unique set of proteins, allowing the cell to regulate spatially its catalytic activities. This requires an efficient system for directing each newly synthesized protein to its correct functional location within the cell.

The targeting signals are ultimately encoded in the protein sequence, and these signals take many forms. Signals for protein import into mitochondria, for example, lack any obvious sequence homology but tend to form amphipathic α-helices or β-sheets, which allow them to bind to the mitochondrial membrane, thereafter specific recognition occurs (Hartl and Neupert, 1990, Baker and Schatz, 1991). Similarly, NH₂-terminal leader peptides, which direct proteins into the ER of eukaryotic cells and function in translocation through the plasma membrane in prokaryotes, also lack highly specific primary sequence determinants. Here more general physical properties such as hydrophobicity and charge are important for function (von Heijne, 1985). Conversely, targeting of some proteins to peroxisomes occurs via a fairly well-defined signal consisting of three amino acid residues at the COOH terminus. The tripeptide SKL at the COOH terminus of firefly luciferase was shown to direct this protein into peroxisomes (Gould et al., 1987). Various substitutions in this tripeptide signal have been made, some of which are functional in peroxisomal import (Subramani, 1992).

The default route for soluble proteins in the secretory pathway is to the cell surface; i.e. they will be secreted if they lack any specific signals. Soluble ER proteins in Saccharomyces cerevisiae contain the COOH-terminal tetrapeptide HDEL, which allows them to be retained in the ER (Pesch, 1989). This is an example of a very specific signal, and recycling of HDEL-tagged proteins to the ER in yeast is mediated by a receptor, Erd2p (Lewis et al., 1990).

In mammalian cells, lysosomal targeting is mediated by the mannose 6-phosphate receptor and, ultimately, by the recognition of the structural elements of the lysosomal proteins by the phosphotransferase (Baranski et al., 1990). Although the yeast vacuole is equivalent to the lysosome of higher eukaryotes in many respects, no mannose 6-phosphate-mediated sorting mechanism has been found in yeast. The soluble vacuolar hydrolases proteinase A and carboxypeptidase Y (CPY) of S. cerevisiae contain vacuolar targeting information in their NH₂-terminal propeptides (Johnson et al., 1987, Valls et al., 1987, Klionsky et al., 1988). The sorting of proteinase A and CPY was suggested to be receptor-mediated since overexpression resulted in mislocalization (Rothman et al., 1986, Stevens et al., 1986). Indeed, the sorting receptor for CPY was recently identified as the VPS10 gene product (Marcusson et al., 1994). The information necessary and sufficient for vacuolar localization of CPY is encoded by a sequence containing the tetrapeptide Gln²⁴-Arg-Pro-Leu²⁷ near the NH₂-terminus of the precursor (Valls et al., 1990). The information for targeting of proteins to the vacuole of plant cells may also be found in short peptide domains (Chrispeels and Raikhel, 1992), but QRPL is the best characterized vacuolar targeting signal so far and is therefore often used as a basis for defining and comparing with other potential vacuolar targeting signals. None of the other known vacuolar proteins in yeast contain a QRPL sequence. However, since only mutations in the QRPL signal abolishing its function have been defined, it is difficult to deduce a consensus sequence for comparison with other systems.

Using degenerate oligonucleotides we have performed extensive mutagenesis on the part of the PRC1 gene encoding the QRPL signal and determined the efficiencies of sorting by immunoprecipitation of newly synthesized CPY in pulse-chase experiments. We found that many mutant forms of the QRPL...
signal are indeed able to sort pro-CYP correctly to the vacuole. Furthermore, we analyzed the importance of the distance of the QRPL signal from the NH2 terminus of the propeptide and found that insertion of up to four amino acid residues did not affect the sorting efficiency seriously. These data together suggest a surprisingly low level of requirement of sequence conservation for function of the recognition of the sorting receptor.

EXPERIMENTAL PROCEDURES

Strains, Media, and Materials—Yeast strains J HRY20–2C.I3 (MATa ura3-52 leu2-3,112 his3-d120 apr1::H153; Vals et al. (1987)) was used for expression of wild-type and mutant PRC1 alleles. Strain SEY6210 (MATa ura3-52 leu2-3,112 his3-d120 trpl-a901 lys2-801 suc2-23; Robinson et al. (1988)) was used for expressing the CPY
invertase fusion. Escherichia coli cultures were grown in LB media (Sambrook et al., 1989). Yeast cultures were grown in standard YPD and SC media (Sherman, 1991). Restriction endonucleases, DNA polymerase I (Klenow fragment), and T4 DNA ligase were from Promega. Plasmid DNA was from Stratagene. Deoxyribonucleotides were from Boehringer Mannheim. Zymolyase was from Seikagaku (Japan). A mixture containing 35S-labeled methionine and cysteine (NEG-072 EXPRE35S) from DuPont was used for in vivo protein labeling. Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer according to the instructions of the manufacturer. Oligo-
nucleotides were annealed on Sephaldex G-200 columns equilibrated in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. Polymerase chain reaction was performed using a DNA thermal cycler from Perkin-Elmer.

Insertion of the stuffer fragment eliminating the QRPL sequence using a cassette mutagenesis procedure and the resulting mutant protein is called aBCd-CPY. A fragment containing this mutant allele was subcloned into pRS315 (a plasmid carrying the CEN vector and the 2μ autonomous replication system) following digestion with XbaI and SmaI.

Plasmid pFL1945 was made from pLV9 (containing PRC1 on a URA3 CEN vector; Vals et al., 1987) by destroying the XhoI site with DNA polymerase I, Klenow fragment, and dNTPs. A polymerase chain reaction fragment was generated extending from the CiaI site upstream of PRC1 to the XbaI site in the PRC1 prorogen. The downstream primer contained three mismatches resulting in a mutation in codon 19 from AAG to CGA, creating a nonsense codon. Amplification was performed using a DNA thermal cycler from Perkin-Elmer.

RESULTS

Mutations at Positions 23–28—In the initial screen we wished to determine the degree of sequence conservation required for efficient recognition of the vacuolar sorting determinant of pro-CYP by its receptor, Vps10p. Thus, various mutant plasmid libraries were constructed covering the region containing the QRPL sequence using a cassette mutagenesis procedure. PRC1 mutant alleles were expressed from centromere plasmids in a Δprc1 yeast strain. To facilitate the mutagenesis, a novel XhoI site was introduced into the gene (Fig. 1). This XhoI site could be used in combination with the naturally occurring XbaI site just downstream of QRPL to replace the wild-type sequence with synthetic oligonucleotides. The mutation that created the XhoI site led to a K19R substitution in the presence of CPY. As expected, this conservative change did not affect translocation of the precursor or precursor cleavage. Furthermore, since the mutant residue was removed by the latter process, there were no effects on further transport or processing.

Mutations were verified by DNA sequencing.
As an initial approach we wished to isolate mutants in the QRPL signal that were proficient in sorting. Thus, libraries of mutant plasmids were constructed at each of the positions Leu$^{23}$ through Leu$^{27}$ (pools Xaa$^{23}$ through Xaa$^{27}$) and more than 500 individual E. coli transformants were obtained from each oligonucleotide mixture. Cells from all transformant colonies of each ligation were pooled, and plasmid DNA was isolated from the pools and used for transformation of a $\Delta prc1$ yeast strain. Mutants were characterized according to two criteria: 1) the presence of intracellular CPY activity, determined by a plate overlay assay, and 2) secretion of CPY, detected by colony immunoblots. Mutants strongly deficient in sorting, as well as non-sense mutants, were not expected to give intracellular activity, while missorting mutants stain positive in the immunoblot. From each collection, 100 transformants were replica-plated and tested according to both criteria. This gave an initial indication as to the specificity of the sorting signal at each position, as well as the frequency of nonsense and frame-shift mutations that would lead to absence of both activity and antigen. The number of colonies without CPY production varied between 5 and 7% in all collections, which corresponds to the expected frequency of stop codons. The most important result of this initial screen was that only two of the 100 yeast Xaa$^{24}$ transformants showed a wild-type phenotype. Screening of the Xaa$^{23}$, Xaa$^{25}$, and Xaa$^{27}$ pools showed a higher frequency (5–15%) of plasmids that gave rise to a wild-type or quasi-wild-type phenotype, while all plasmids from the Xaa$^{25}$ pool appeared to give a wild-type phenotype. Sequencing of the plasmid DNA from the two positive clones of the Xaa$^{24}$ pool showed that the wild-type phenotype was in both due to a Gln codon.

All plasmids giving what appeared to be a wild-type phenotype in the plate assays were sequenced, and to get a broader view of the specificity, several randomly chosen plasmids were selected as correctly sorting in the plate assays of the random mutants at this position. This mutation does not change QRPL or its direct effect on sorting. Most severe is the R25D mutation, which results in secretion of 48% of the newly synthesized pro-CPY. All the other mutations have a sorting efficiency between 74 and 95%. This is in good agreement with the large number of mutations (Asn, Asp, Glu, Gly, Lys, and Val) scored as wild-type or quasi-wild-type in the initial plate screen.

Mutations in Pro$^{25}$ give rise to a wide spectrum of phenotypes. P26R leads to missorting of almost all of the newly synthesized pro-CPY, whereas the P26S mutation still localizes 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole. Mutants with hydrophobic amino acid residues (Phe, Val, Leu, Ala) at this position show 73% of the pro-CPY to the vacuole.

**Fig. 2. Immunoprecipitation of CPY antigen from selected mutants.** Cells expressing three mutant $prc1$ alleles and the wild type (WT) were labeled for 20 min with $^{35}$S-labeled amino acids and chased with unlabeled amino acids and chased for 60 min. Intracellular (I) and extracellular (E) material were separated, and CPY antigen was immunoprecipitated. The labeled precipitates were subsequently subjected to 8% SDS-polyacrylamide gel electrophoresis. Within the duration of the chase period all CPY reaches its final destination.

In general, mutations in Arg$^{25}$ do not appear to have a strong effect on sorting. Most severe is the R25D mutation, which results in secretion of 48% of the newly synthesized pro-CPY. All the other mutations have a sorting efficiency between 74 and 95%. This is in good agreement with the large number of mutations (Asn, Asp, Glu, Gly, Lys, and Val) scored as wild-type or quasi-wild-type in the initial plate screen.

Replacing the codon for Gly$^{28}$ by random DNA sequence was not possible using random substitutions since this codon forms part of the XbaI site used for ligation of the mutagenic oligonucleotides (Fig. 1). Consequently, directed mutations were introduced to exchange Gly$^{28}$ with arginine, phenylalanine, or aspartic acid residues. Pulse-chase experiments showed that even these radical changes had limited effect, the strongest being Asp$^{28}$, which gives missorting of 21% of the newly synthesized pro-CPY (Fig. 3).
environment but increases the distance to the amino terminus of the propeptide. Quantitative immunoprecipitation showed that there was no significant effect of this mutation on pro-CPY sorting (Table I).

In a more radical approach, four extra codons were introduced between codons 22 and 23. Each extra codon started with adenine followed by two random nucleotides to avoid stop codons. Five plasmids resulting from this mutagenesis were sequenced (Table I), and the sorting efficiencies of the respective CPY mutant proteins were determined in pulse-chase experiments. One of the mutants secretes 25% of the total amount synthesized, which is significantly more than the wild type. The secretion of the other four mutant CPY proteins, however, is only slightly higher than that of the wild type.

CPY Mutant Proteins Do Not Poison the Receptor—Missorting of pro-CPY with altered targeting sequence is most cases likely to be due to inability to interact functionally with the receptor. This has been directly shown by failure of the Q24K to chemically cross-link to the receptor (Marcusson et al., 1994). Mutations that result in pro-CPY molecules with a too high affinity for the receptor would potentially also lead to missorting. Inability to be released from the receptor in the acceptor compartment would thus lead to poisoning of the receptor. It has been shown that mutations Q24K and L27S do not poison the receptor (Valls et al., 1990). To identify putative receptor-poisoning mutants we used a fusion protein consisting of the first 156 amino acid residues of prepro-CPY and the SUC2-encoded enzyme invertase. Invertase is normally secreted while the CPY-invertase hybrid protein is efficiently targeted to the vacuole (Johnson et al., 1987). Plasmid DNA from the four libraries containing QRPL mutations was introduced into Dsuc2 cells containing the plasmid pFV127 directing the production of the CPY-invertase hybrid protein. Cells expressing a PRC1 allele that would lead to poisoning of the receptor were expected to secrete the pro-CPY-invertase fusion protein and show extracellular invertase activity. 500 yeast transformants from each of the Xaa24–Xaa27 libraries were tested in a plate overlay assay for extracellular invertase activity. All were negative.

In parallel to this approach we tested mutations P26D, L27A, L27G, and L27R, which exhibited the strongest missorting phenotypes, using an approach very similar to that described by Valls et al. (1990), analyzing the competition between aberrant glycoforms of CPY and the QRPL mutant forms relative to the total amount of labeled CPY, intracellularly and extracellularly, after a 20-min 35S pulse followed by a 60-min chase. Shaded bars indicate mutants that have been characterized in this study, while those indicated by hatched bars are from Valls et al. (1990). wt, wild type.
in immunoprecipitation experiments (not shown). This approach also suggested that receptor poisoning is a phenomenon that cannot be brought about by mutation of a single residue in the QRPL signal. Thus, we find it unlikely that single amino acid substitutions can lead to poisoning of the receptor.

**Discussion**

The ligand-receptor pair, which is central to the present discussion, represents the first and best characterized member of a new class of intracellular sorting mechanisms. It has long been known that the signal for pro-CPY sorting to the vacuole resides in the proregion and that a sequence containing Gln24-Arg-Pro-Leu27 is both necessary and sufficient for this function (Valls et al., 1987, 1990; Johnson et al., 1987). Through the use of genetic screens, this observation eventually led to the identification of the VPS10 gene, which encodes the receptor directly involved in QRPL recognition. The VPS10 gene product (Vps10p) is a very large transmembrane protein (1577 amino acid residues) with a short COOH-terminal cytoplasmic tail (164 amino acid residues). In cell fractionation as well as functional studies, it localizes to the distal Golgi apparatus together with the Kex2 protease (Marcussson et al., 1994, Graham et al., 1991). Chemical cross-linking experiments have shown that Vps10p interacts specifically with the QRPL signal, both in a pro-CPY context and in an invertase fusion context (Marcussson et al., 1994). Mutations that resulted in missorting of pro-CPY had been found in each of the QRPL residues (Valls et al., 1990), and it was directly shown that the Q24K mutant protein did not chemically cross-link to Vps10p (Marcussson et al., 1994). However, the level of sequence conservation required for productive pro-CPY-Vps10p interaction has not been investigated. Since the QRPL signal has been described in such detail it has to some extent been regarded as a paradigm for comparison to other non-carbohydrate-dependent lysosomal/vacuolar sorting systems. It has been suggested that the QRPL signal might be a part of a larger "consensus sequence" for lysosomal sorting both in mammals and yeast (McIntyre et al., 1994). The original screen would not address the validity of such a consensus since the mutants were identified by their inability to confer vacuolar sorting to pro-CPY, i.e. no functional mutants were isolated (Valls et al., 1990). This has also hampered the identification of other putative QRPL sequences by homology searches, as the significance of individual residues was not known. In the present work we have attempted to approach a consensus sequence for the specificity of the receptor-ligand interaction.

One of the results of the present study is the identification of the pivotal role of Gln24 in the sorting process. All mutations at this position showed severe missorting phenotypes, and we failed in our attempts to isolate, by activity stain and immunoblotting, mutants that were able to functionally substitute for Gln24. We therefore conclude that this amino acid residue is essential for the proper recognition of the targeting element. There seems to be a similar importance for the structural conservation of Leu27, although several hydrophobic residues (Phe, Val, and Ile) were functional. Because of the mutational approach taken, we cannot exclude the possibility that tryptophan or methionine residues, encoded by rare codons, might also function.

Although missorting mutations can be found at positions Arg25, Pro26, and Gly28, the nature of the residues at these positions is clearly of lesser importance. The discussion of the phenotypes of these mutants to some extent depends on the definition of missorting. One should realize that pro-CPY sorting is never 100% efficient; we typically find around 5% of the newly synthesized wild-type pro-CPY to be missorted. This might be due to problems of stoichiometry at the site of inter-

action, but it might also be due to an inherent lack of affinity. It is conceivable that CPY might also, under some conditions, be beneficial for extracellular peptide hydrolysis. Thus, there may not have been strong evolutionary pressure for 100% sorting efficiency.

In any event, the substitutions for Arg25 in several cases lead to a clearly wild-type phenotype. However, it is not easy to rationalize these phenotypes from structural considerations since not only the conservative change to lysine but also radical changes to the hydrophobic amino acids leucine and valine and the polar glutamine fail to affect sorting. In addition, most other mutations obtained have only fairly weak missorting phenotypes, less than 30% being secreted in most strains. Only a substitution by aspartate appears to affect sorting strongly. Most of the mutants characterized at this position were selected on the basis of their proficiency in sorting. Thus, the relatively small number of strong missorting mutants reflects the selection procedure employed.

Mutations leading to amino acid substitutions for Leu23 had a similar effect (Fig. 3). On immunoblots, no mutants could be identified that showed severe missorting. Thus, randomly chosen mutations were tested in immunoprecipitations, and only weak phenotypes were found. These findings support the notion that Leu23 is not very important.

Proline is important for specific tertiary structures in many protein contexts. It is therefore surprising that many of the randomly selected mutations at position 26 only had limited effect. There appears to be a correlation between charge and missorting phenotype and a clear preference for hydrophobic residues. The phenotype of the Asp28 mutant (21% missorting) could suggest that Gly28 is just as much a part of the sorting signal as Phe26. Considering that glycine also has unique structural features, one might have expected more severe missorting phenotypes. As these two examples show, there is a remarkable insensitivity toward the tertiary structural environment of the QRPL signal. This notion is supported by the observation that fairly short fusions (30 peptidase residues) of the QRPL signal to invertase are able to direct this secreted protein to the vacuole (Johnson et al., 1987). Furthermore, the deletion of large fractions of the propeptide downstream of Asp38 had no effect on sorting (Ramos et al., 1994). Indeed, the combined structural and biochemical analysis of the propeptide suggests that it may have a highly flexible and dynamic structure (Sørensen et al., 1993).

Considering the essential role of the propeptide in folding (Winther and Sørensen, 1991; Ramos et al., 1994), mutagenesis in the propeptide could result in aberrantly folded pro-CPY molecules, and these misfolded molecules might therefore not be recognized by the sorting receptor. It has been shown that mutants having amino acids 27–31 deleted are folded correctly and exit from the ER with normal efficiency (Valls et al. 1987). This suggested that changes in this part of the propeptide would not seriously affect folding. We examined one representative mutant for each position with shorter pulse and chase times and observed maturation half-lives shorter than 15 min (data not shown). The insertion mutants containing four extra residues, however, did show a minor folding defect suggested by a half-time of ER exit of about 30 min. This somewhat extended half-time of maturation also resulted in a small amount, less than 10%, of pro-CPY in the intracellular fraction after a 1-h chase. In all the other experiments there was no detectable pro-CPY left in the intracellular fractions after a 1-h chase. After the 20-min labeling the relative amount of CPY in the proform is typically 20–40%. The minimal detection limit is 2.5%. This also shows that the half-time of maturation for all the single-amino acid mutants is less than 15 min. One should
also bear in mind that it is unlikely that misfolded pro-CPY would escape the ER. Indeed, deletions in the propeptide that result in reduced efficiency of folding do not result in missorting (Ramos et al. 1994). Finally, the folded mature domain of CPY is not necessary for recognition by the receptor since CPY-invertase hybrid proteins containing as little as 30 amino acid residues of CPY are sorted efficiently (Johnson et al. 1994). The QRPL signal does not conform to a SXX+XL consensus (where + is a positively charged residue and X is any amino acid residue) as suggested previously (McIntyre et al., 1994). The initial S is supposed to correspond to Ser-22 in pro-CPY, but this residue is not important, while Gln-24 is. Apart from the apparently essential function of Gln-24, one should probably be cautious with defining a consensus. Although there are clear tendencies to suggest specificity for hydrophobic residues at position 27, it is difficult to rationalize the missorting phenotypes for the other residues. It is somewhat surprising conclusion of the present work is that there does not appear to be a conventional “consensus sequence” for ligands of Vps10p. It will thus be difficult to identify ligands by sequence alignment and, in a comparison across species, this problem would be further enhanced by slight changes in specificity likely to occur even between related ligand-receptor pairs.

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