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Overproduction-induced mislocalization of a yeast vacuolar protein allows isolation of its structural gene

(proteinase A/lysosome/proteolytic processing/protein localization/immunological screening)

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ABSTRACT **Using an immunological screening procedure that allows the detection of yeast cells aberrantly secreting vacuolar proteins, we have isolated a cloned DNA fragment containing the structural gene for the vacuolar enzyme proteinase A (PrA; EC 3.4.23.6). A large portion of PrA is misdirected to the cell surface in cells harboring the PrA structural gene on a multicopy plasmid. This mislocalized PrA traverses the late stages of the secretory pathway and differs slightly in apparent molecular weight from the vacuolar form. A deletion in the genomic copy of the PrA structural gene eliminates immunoreactive PrA as well as the enzymatic activities of at least three other vacuolar hydrolases. In the case of the vacuolar enzyme carboxypeptidase Y (EC 3.4.16.1), the lack of activity is due to the absence of proteolytic activation of the zymogen. Thus, PrA may be required for *in vivo* processing of a number of yeast vacuolar hydrolases.**

Newly synthesized proteins of the yeast lysosome-like vacuole transit early portions of the secretory pathway prior to delivery to this organelle (1). These proteins presumably carry localization signals that are recognized by vacuolar localization machinery associated with this portion of the secretory pathway. Molecular cloning and *in vitro* mutagenesis of *PRC1*, the gene encoding the vacuolar protein carboxypeptidase Y (CPY; serine carboxypeptidase, EC 3.4.16.1) has allowed the identification of putative vacuolar localization signals carried by this protein (2). These analyses indicate that determinants necessary for delivery of this protein to the vacuole reside within the N-terminal propeptide of the CPY precursor (2). Since other vacuolar proteins are also synthesized as larger precursors (3), it is of interest to determine whether the propeptides of vacuolar precursor proteins generally carry localization determinants for such proteins.

The vacuolar enzyme proteinase A (PrA; *Saccharomyces* aspartic proteinase, EC 3.4.23.6) is synthesized as a 52-kDa glycosylated precursor (3). The active form of the protein resides in the vacuole as a 42-kDa glycoprotein containing 6 kDa of asparagine-linked carbohydrate (3, 4). Although no *in vivo* function for the enzyme has been determined, two classes of mutations that abolish PrA activity have been described. Mutations in the putative PrA structural gene (*PRA1*) specifically affect PrA: *pra1* mutants are defective in PrA activity but show normal levels of other vacuolar enzyme activities (5). In contrast, a number of mutations have been reported (6) that result in a pleiotropic phenotype, affecting the activities of PrA and several other vacuolar hydrolases. Among the mutants exhibiting a pleiotropic phenotype, *pep4* mutants (7) are best characterized. It is likely that the *PEP4* gene encodes a function required for proteolytic processing of vacuolar protein precursors, since the unprocessed form of at least one vacuolar protein, CPY, accumulates (8) within vacuoles of *pep4* cells (1).

To examine the structure of vacuolar localization signals on PrA and the role PrA may play in processing of vacuolar zymogens, we have cloned the PrA structural gene. We have obtained this clone by using an immunological screening procedure that allows detection of cells overproducing PrA. In this report we describe the mislocalization of PrA in cells overproducing the protein and describe the pleiotropic phenotypes observed in cells completely deficient for PrA.

MATERIALS AND METHODS

Strains and Materials. Yeast strains JHRY1-5Dα (*his4-519, ura3-52, leu2-3, leu2-112, trp1, pep4-3*) and JHRY20-2Ca (*his3-Δ200, ura3-52, leu2-3, leu2-112*) were constructed by standard genetic techniques. Strains SF838-1Dα (*ade6, his4-519, ura3-52, leu2-3, leu2-112, pep4-3*) and ISY1-7Ba (*his4, leu2-3, leu2-112, ura3-52, suc2-Δ9, pho80*) were obtained from R. Schekman. Growth of yeast cells for enzymatic assays and immunoprecipitations was as described elsewhere (9). Enzymes used in recombinant DNA manipulations were from Bethesda Research Laboratories and New England Biolabs. Carrier-free $H_2{}^{35}SO_4$ was purchased from ICN. Yeast PrA, phosphoglycerate kinase, and reagents used in enzyme assays were from Sigma. IgGsorb was from The Enzyme Center (Boston, MA). Nitrocellulose filters (0.45-μm pore diameter) were from Millipore. Reagents used in immunoblots were obtained from Bio-Rad.

Antibodies. Affinity-purified CPY antibody was prepared as described previously (1). Antiserum to PrA and phosphoglycerate kinase were prepared as described for CPY (1). To remove carbohydrate-reactive antibodies from the PrA antiserum, the serum was adsorbed twice to whole yeast cells by suspending 0.5 g of cells (wet weight) with 20 ml of serum and sedimenting the cells.

Immunoblotting. Transformed yeast colonies were replica-plated onto minimal agar plates containing the required nutrients, and the replicas were overlaid with prewetted 85-mm-diameter nitrocellulose filters. After incubation at 30°C for 12–24 hr, the filters were removed and adhering cells were rapidly washed off with 20 mM Tris·HCl, pH 7.5/500 mM NaCl. The filters were then treated according to the protocol supplied with the Bio-Rad Immuno-Blot assay kit, substituting Carnation nonfat dry milk for gelatin as a source of nonspecific protein.

Amino-Terminal Analysis of PrA. Electrophoretically pure yeast PrA from Sigma was dialyzed against water, lyophilized, and analyzed on a Beckman 890M liquid phase sequencer at the University of California at Davis Protein Research Laboratory.

Recombinant DNA Procedures. All cloning manipulations were performed by using standard techniques (10). Plasmid

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Abbreviations: CPY, carboxypeptidase Y; PrA, proteinase A; kb, kilobase(s).

pTS18 was constructed by inserting a 4.1-kilobase (kb) DNA fragment carrying the complete PrA structural gene into YCp50, a single-copy centromere-containing plasmid (Fig. 2). The deletion in the PrA structural gene was constructed by replacing the 1.2-kb *Hin*dIII–*Hin*dIII fragment within a subclone of the gene with the 1.1-kb *URA3* fragment from YEp24, creating the plasmid pTS15. The resulting substitution was integrated into the genome by the procedure of Rothstein (11): an *Xho* I–*Eco*RI fragment from pTS15 was used to transform yeast, and stable uracil-independent transformants were selected. Southern analysis indicates that the integrated *URA3* gene maps to the genomic locus of the PrA structural gene (12). DNA sequence analysis of the PrA structural gene is described elsewhere (12).

Preparation of Crude Extracts and Enzyme Assays. Clarified crude extracts were prepared from glass-bead-broken cells as described in ref. 12. CPY (9), alkaline phosphatase (EC 3.1.3.1) (13), proteinase B (EC 3.4.21.48) (7), and total protein (14) were assayed as described elsewhere. PrA was assayed according to ref. 15, using denatured hemoglobin as prepared in ref. 7.

Immunoprecipitations. Extracts corresponding to intracellular, periplasmic, and medium fractions were obtained from $H_2{}^{35}SO_4$-labeled yeast cells and immunoprecipitated as described elsewhere (9). Total extracts were obtained and immunoprecipitated as described in ref. 12. All precipitations were carried out in antibody excess. Gel electrophoresis, fluorography, and quantitation of bands were performed as described elsewhere (9).

RESULTS

Immunoblotting Procedure Detects Secreted Vacuolar Proteins. To identify yeast cells that mislocalize vacuolar proteins to the cell surface, we have developed an immunoblotting procedure that allows the detection of extracellular but not internal antigens. Yeast cells containing multiple copies of the *PRC1* gene carried on a 2-μm ("2μ") plasmid (pTSY7) secrete immunoreactive CPY (9), which can be detected as a dark spot on a nitrocellulose filter when CPY antibody is used in the screening procedure (Fig. 1). The same yeast strain carrying a plasmid lacking *PRC1* sequences (YEp24) does not result in a positive reaction on the nitrocellulose filter (Fig. 1) because such cells retain all CPY in their vacuoles.

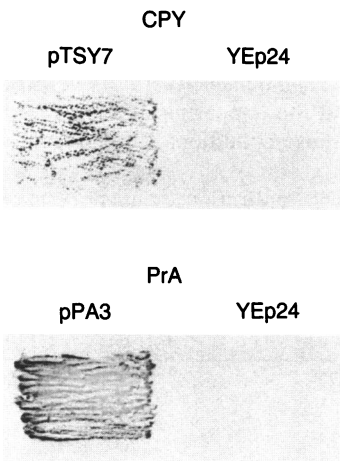

FIG. 1. Detection of extracellular vacuolar protein antigens by using a nonlysing immunoblotting procedure. Nitrocellulose filters were placed onto plates containing freshly patched yeast cells and subsequently treated with affinity-purified CPY or PrA antibody. pTSY7: strain ISY1-7Ba carrying the *PRC1* gene on a 2-μm plasmid. YEp24: strain ISY1-7Ba (*Upper*) or JHRY20-2Ca (*Lower*) carrying a 2-μm plasmid lacking sequences encoding vacuolar proteins. pPA3: strain JHRY20-2Ca carrying the PrA structural gene on a 2-μm plasmid.

Identification of a PrA-Secreting Clone. Because a significant fraction of CPY is secreted when the *PRC1* gene product is overproduced in cells carrying multiple copies of this gene, we reasoned that yeast cells harboring the structural gene for PrA on a multicopy plasmid might similarly secrete PrA. This would result in a positive reaction in the immunoblotting screen when the filters are treated with PrA antiserum. To identify putative PrA-encoding clones, yeast strain JHRY1-5Dα was transformed with plasmid DNA from a YEp24-based clone bank (16). Between 500 and 2000 transformant colonies per plate were replica-plated and immunoblotted with PrA antiserum. In a screen of approximately 14,000 uracil-independent transformants a single dark spot was observed on one nitrocellulose filter. The colony corresponding to this spot was purified and plasmid DNA was isolated from the purified yeast clone. Reintroducing the purified plasmid (pPA1) into yeast resulted in the apparent secretion of PrA by immunoblot analysis. Thus, pPA1 carries a DNA sequence that causes yeast cells to secrete a portion of their PrA.

Analysis of the DNA Fragment that Causes PrA Secretion. Restriction mapping reveals that pPA1 contains an 11-kb insert of yeast genomic DNA (Fig. 2). To define the limits of the DNA segment resulting in PrA secretion, various subclones of pPA1 were constructed in YEp24, and yeast transformants (*PEP4* or *pep4*) carrying the subcloned plasmids were examined for secretion of PrA (Fig. 2). An example of an immunoblot screen with one subclone (pPA3) is shown in Fig. 1. The results of these analyses indicate that the DNA segment which causes PrA secretion crosses the right-hand *Cla* I site and is contained within the 3.9-kb *Stu* I–*Bam*HI fragment of pPA3 (Fig. 2).

pPA1 Encodes PrA. If the cloned insert described above indeed encodes PrA, then multiple copies of the plasmid-borne structural gene should result in overproduction of PrA. As shown in Table 1, cells containing pPA3 exhibit about an 8-fold higher level of intracellular PrA activity and a 16-fold higher level of total immunoprecipitable PrA antigen (including PrA in the medium) than the same strain carrying the parent 2-μm plasmid YEp24. Only PrA is dramatically overproduced in cells containing pPA3; the activities of other vacuolar proteins (CPY and alkaline phosphatase) are not elevated, and proteinase B activity increases by a small amount (≈50%, Table 1).

We have compared the sequence of the amino-terminal 12 amino acids of the mature PrA polypeptide with the amino acid sequence predicted from DNA sequence analysis of a portion of the pPA1 insert. Within the translated DNA sequence, there is a 12 amino acid stretch that is identical to the amino terminus of the mature protein (Fig. 2). In addition, the remainder of the amino acid sequence predicted from the DNA sequence is identical with that of the mature PrA polypeptide (T. Dreyer, personal communication). Taken together, these observations indicate that the yeast genomic insert in pPA1 indeed includes the structural gene for PrA.

Mislocalization of PrA to the Periplasm and Medium upon Its Overproduction. The quantity and characteristics of secreted PrA in cells overproducing the protein were examined by immunoprecipitating the protein from intracellular, periplasmic, and medium fractions isolated from $H_2{}^{35}SO_4$-labeled yeast cells. The resulting immunoprecipitates were examined on a fluorogram of a polyacrylamide gel (Fig. 3*A*). In cells carrying one or two copies of the PrA structural gene no immunoreactive PrA is detectable in the secreted fractions (periplasm and medium). In sharp contrast, cells harboring the PrA structural gene on a multicopy (2-μm) plasmid (pPA3) secrete over half of the total PrA synthesized, primarily to the medium. Under conditions in which a large

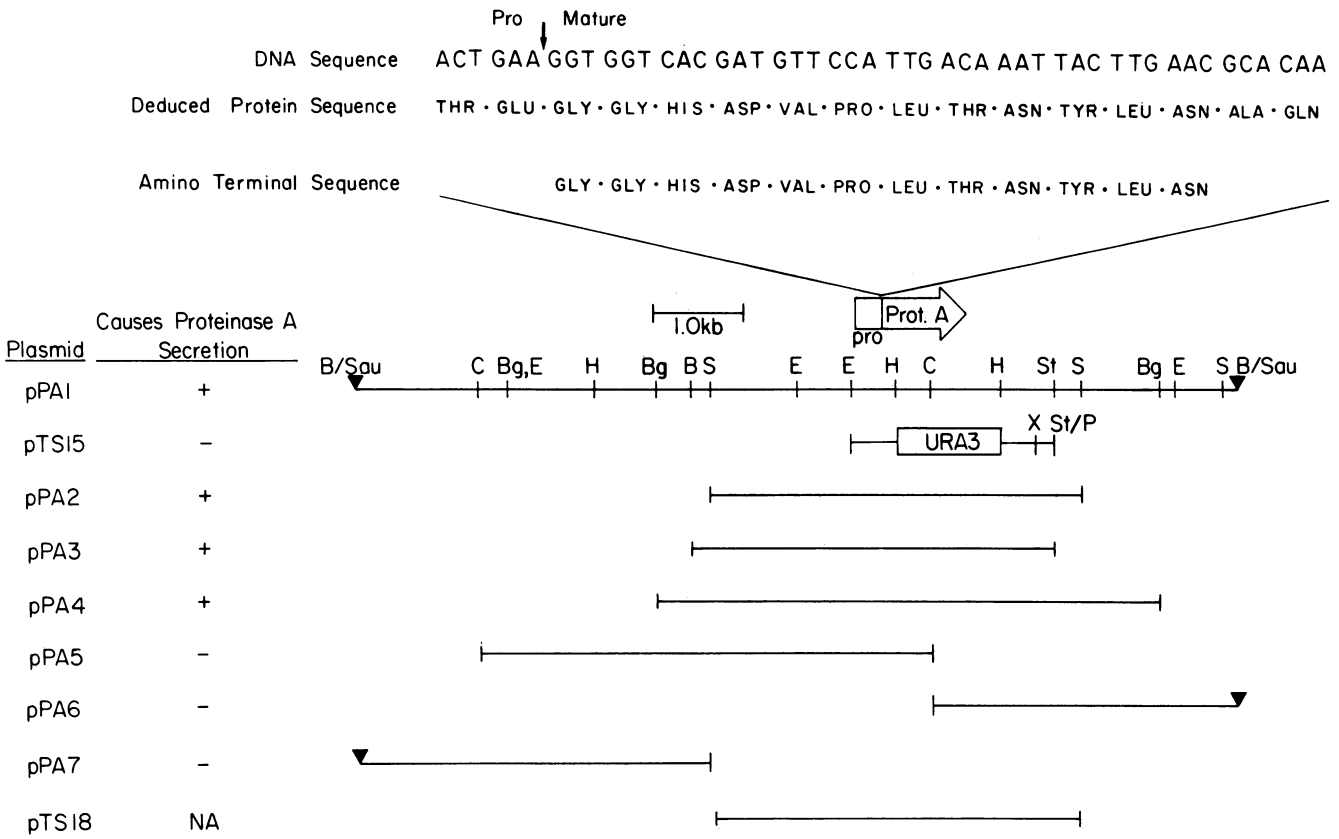

FIG. 2. Restriction map of the yeast genomic insert in plasmid pPA1. At the top of the figure are indicated the protein sequence around the amino terminus of the mature polypeptide deduced from DNA sequence analysis (12) and amino-terminal analysis of the first 12 amino acids of the mature PrA polypeptide. Pro indicates the carboxyl-terminal residues of the propeptide. The diagram below the restriction map indicates the region deleted and replaced by *URA3*-encoding sequences in the PrA-deficient plasmid (pTS15) and the various subclones of the PrA structural gene used to analyze the extent of the sequences responsible for secretion of PrA. The presence or absence of secreted PrA in JHRY20-2Ca transformants containing the various subcloned fragments was assessed by using a blotting procedure similar to that shown in Fig. 1. B, *Bam*HI; Bg, *Bgl* II; C, *Cla* I; E, *Eco*RI; H, *Hin*dIII; P, *Pvu* II; Sau, *Sau* IIIA; S, *Sal* I; St, *Stu* I; X, *Xho* I; ▼, endpoints of genomic DNA insert; NA, not applicable.

portion of PrA is secreted, no CPY antigen or vacuolar alkaline phosphatase activity is found to be secreted (data not shown). The migration of the secreted form of PrA does not coincide with the intracellular form of the protein; it migrates between the mature PrA protein and the faster-migrating of the two PrA precursors (p1 and p2) that we can detect (Fig. 3*B*).

Three lines of evidence support the hypothesis that the presence of extracellular PrA antigen is not a result of cell lysis. First, the form of PrA secreted does not comigrate with the internal form of PrA. Furthermore, antibody to phosphoglycerate kinase, a cytoplasmic enzyme, does not precipitate significant amounts of that protein from the secreted fractions under conditions in which a large portion of PrA is secreted (data not shown). Finally, the late-acting *sec1-1* mutation (17) blocks delivery of overproduced PrA to the cell surface at the nonpermissive temperature (data not shown). Under these conditions, the portion of PrA corre-

Table 1. Quantitation of PrA secretion, total PrA antigen, and enzymatic activities of vacuolar proteins in strains carrying zero, one, or multiple copies of the PrA structural gene

Strain	PrA activity, U/mg*	Relative PrA antigen	% PrA antigen secreted	CPY activity, mU/mg	Alkaline phosphatase activity, mU/mg	Proteinase B activity, mU/mg
JHRY20-2Ca/YEp24	9.6	1.0	<1	174	208	9.6
JHRY20-2Ca/pTS18	22.3	2.3	<1	161	183	11.1
JHRY20-2Ca/pPA3	81.5	16.2	54.5	110	170	13.9
JHRY20-2Ca Δ†	<0.1	—	ND	3	35	0.5
SF838-1Dα/YEp24	0.4	—	ND	3	36	0.2
SF838-1Dα/pPA1	57.9	ND	ND	132	119	20.3

Total and secreted PrA antigen were determined by quantitating bands excised from fluorograms. Relative PrA antigen values were normalized for incorporation of $H_2{}^{35}SO_4$ into protein (1). Percent PrA antigen secreted is expressed as the ratio of PrA antigen in the periplasmic and medium fractions to the PrA antigen in all three fractions. Activities are expressed as milliunits/mg of protein except where noted. Residual alkaline phosphatase activity in JHRY20-2CaΔ and SF838-1Dα/YEp24 is probably attributable to the cytoplasmic enzyme (7). —, None detectable; ND, not determined.

*Expressed as units/mg of protein. Does not include activity of medium.

†Carries a deletion in the PrA coding sequences as diagrammed in Fig. 2.

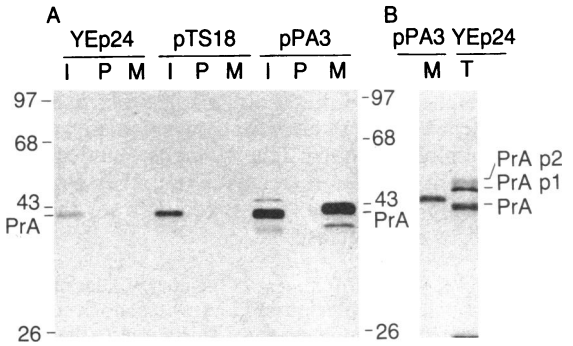

FIG. 3. NaDodSO$_4$/PAGE analysis of intracellular and secreted PrA. PrA was immunoprecipitated from extracts of strain JHRY20-2Ca containing the indicated plasmids. (*A*) Fluorogram of a 10% polyacrylamide gel; (*B*) fluorogram of an 8% polyacrylamide gel. Cultures were pulsed with $H_2{}^{35}SO_4$ for 20 min and chased for 45 min in 10 mM sulfate (*A* and left lane of *B*), or pulsed for 20 min without chasing (right lane of *B*). The positions of the two precursor forms of PrA (p1 and p2) and mature PrA are indicated in *B*. Molecular masses of protein standards are given in kDa. Fractions are I, intracellular; P, periplasmic; M, medium; and T, total extract. The minor high molecular weight bands (I lanes) are of unknown origin.

sponding to the secreted material in wild-type cells accumulates intracellularly (presumably within secretory vesicles).

PrA Structural Gene Clone Suppresses the Phenotype of a *pep4* Mutant. The *PEP4* gene is required for the activity of several vacuolar enzymes, including PrA (7), and for proteolytic processing of the CPY precursor (8). It has also been reported that cells carrying the *pep4-3* nonsense mutation accumulate PrA precursor of an unusually high molecular weight (8). In contrast, we (Fig. 4) and others (18) detect no immunoreactive PrA in cells carrying the *pep4-3* mutation. Surprisingly, we detect large quantities of immunoreactive PrA in *pep4* cells harboring pPA1; such cells exhibit only fully processed (42-kDa) PrA intracellularly (data not shown). In addition, high levels of PrA activity and wild-type levels of other vacuolar enzyme activities are observed, in sharp contrast to the same *pep4* strain carrying a plasmid (YEp24) that lacks any PrA structural gene sequences (Table 1). These results indicate that high levels of PrA can suppress the pleiotropic phenotype of a *pep4* mutant.

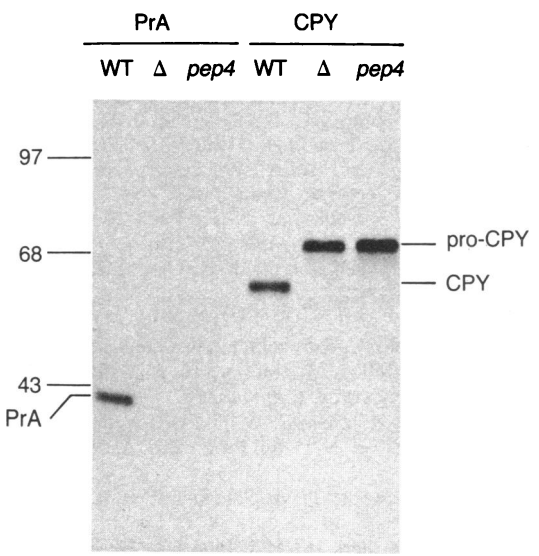

FIG. 4. Immunoprecipitation of PrA and CPY from total yeast cell extracts. All extracts were obtained by glass-bead breakage of labeled cells and were immunoprecipitated with the indicated affinity-purified antibody. The positions of mature PrA, the precursor (pro-CPY) and mature CPY, and protein standards (in kDa) are indicated. WT, wild-type strain JHRY20-2Ca carrying YEp24; Δ, JHRY20-2Ca deleted for the PrA structural gene; *pep4*, strain SF838-1Dα carrying YEp24.

Phenotypes of a Yeast Strain Deleted for the PrA Structural Gene. The phenotype of a PrA deficiency was examined by constructing a deletion in the PrA structural gene and marking the deletion with the *URA3* gene (Fig. 2). This substitution was inserted into the genome of yeast strain JHRY20-2Ca as described in *Materials and Methods*. As shown in Table 1, the resultant clones are deficient in PrA activity, as would be expected for strains carrying a deletion in the PrA structural gene. In addition, no PrA antigen can be immunoprecipitated from these cells (Fig. 4). Since none of the reported mutations in the *PRA1* locus cause cells to be deficient for any vacuolar enzyme activities other than PrA (5), it seemed likely that deletion of the PrA structural gene would result in a nonpleiotropic phenotype. However, deletion of the PrA-encoding gene leads to deficiencies in CPY, proteinase B, and alkaline phosphatase activities as well (Table 1). The nature of the deficiency in CPY activity was examined by immunoprecipitating an extract from the strain deleted for the PrA structural gene with CPY antibody. Wild-type cells exhibit mature (57-kDa) CPY antigen, whereas the isogenic strain deleted for the PrA structural gene accumulates a CPY antigen that comigrates with the 70-kDa precursor form observed in a *pep4-3* strain (Fig. 4). Thus, the PrA-deficient strain fails to process pro-CPY to the mature form.

Intracellular proteolysis is apparently required for sporulation of diploid yeast cells (19); therefore we examined the sporulation proficiency of a diploid homozygous for the deletion of the PrA structural gene. Such diploids were incapable of sporulation (frequency of sporulation less than 1/1000th of that of the isogenic parents carrying wild-type PrA structural genes), consistent with observed pleiotropic effects of a PrA deficiency.

DISCUSSION

Using an immunological technique, we have cloned the structural gene encoding PrA. Evidence that our clone does indeed encode PrA comes from comparison of the PrA amino acid sequence with the translated DNA sequence. In addition, the observations that multiple copies of this gene cause overproduction of PrA activity and that deletion of the gene from the genome causes the elimination of immunoreactive PrA further support this conclusion.

While our work was in progress, an immunoblotting method involving lysed yeast cells was reported (20). The latter procedure allows cloning of a gene in which a mutation eliminating immunoreactive gene product exists. The advantage of our procedure is that a gene can be rapidly cloned from yeast cells in the absence of a mutation in that gene. Because both CPY and PrA are secreted when overproduced, this procedure may be generally applicable to cloning other soluble vacuolar protein structural genes.

A number of mutations in *PRC1* have been isolated by using the immunoblotting technique described here (2). These mutations cause CPY to be secreted instead of being directed to the vacuole and presumably reside within CPY vacuolar localization determinants. Since these *PRC1* mutants synthesize normal quantities of CPY, it is clear that the immunoblotting procedure is capable of differentiating cells secreting vacuolar proteins from normal cells, even when these proteins are not being overproduced. By using the PrA structural gene clone and our immunoblotting screen it should be possible to determine whether PrA shares similarities with CPY in its vacuolar localization determinants.

As has been recently observed for CPY (9), increasing the copy number of the PrA structural gene results in the secretion of a large quantity of the newly synthesized protein (Table 1). One explanation for this observation is that overproduction of vacuolar proteins results in saturation of a step in the process that sorts secretory and vacuolar proteins. Since overproduction of CPY does not result in the secretion of PrA (9), and vice versa, it seems unlikely that missorting of these proteins upon overproduction is due to saturation of a sorting step (e.g., receptor) common to all vacuolar proteins. The observation that PrA is secreted when overproduced is not explained by invoking saturation of a putative extracellular degradation process that destroys missorted PrA, since radiolabeled PrA precipitated from the medium and periplasm is found to be stable during a several-hour chase period (data not shown).

The secretion of PrA is blocked by a *sec1* (late-acting, secretory vesicle-blocked) mutation at the restrictive temperature, suggesting that missorted PrA traverses the last stage of the secretory pathway en route to the cell surface. When PrA is mislocalized to the cell surface its migration on polyacrylamide gels is intermediate between that of the internal precursor and mature PrA forms (Fig. 3*B*). It seems likely that secreted PrA, like secreted CPY (9), does not undergo the extensive outer chain glycosyl modifications characteristic of secretory proteins such as invertase (21). In particular, unlike secreted invertase, secreted PrA migrates as a discrete band. It is conceivable that the difference in apparent molecular weight of the vacuolar and secretory forms of PrA reflects a modification of PrA required for its sorting and that this modification is absent from the secreted form, resulting in its secretion. Further studies are required to determine whether the aberrant mobility of secreted PrA is due to differences in proteolytic processing or some other post-translational modification.

Overproduction of PrA in a *pep4* strain leads to an unexpected phenotype—i.e., the appearance of mature active PrA intracellularly and the presence of other vacuolar enzyme activities. If PrA is capable of weak autoactivation [as has been suggested for proteinase B (22)], overproduction of PrA might allow the accumulation of enough of the mature enzyme to establish an autoactivation cascade. A second explanation for this phenotype is that *PEP4* is the PrA structural gene.

A deletion in the PrA-encoding sequence results in the elimination of PrA activity and immunoreactive protein (Fig. 4). However, the surprising observation that this deletion has a pleiotropic phenotype, eliminating the activities of other vacuolar hydrolases (Table 1) and preventing the proteolytic processing of pro-CPY (Fig. 4), strongly implicates an essential role of PrA in the activation of vacuolar proteins. No *pra1* mutant alleles that exhibit a pleiotropic phenotype have been reported (5), although it has not been demonstrated that *PRA1* indeed encodes PrA. (Due to the unavailability of the *pra1* mutants we have not tested whether the PrA structural gene clone is allelic to *pra1* mutations.) Given our observations that a deficiency in the PrA structural gene leads to inactivation of several vacuolar hydrolases, that cells carrying the *pep4-3* nonsense mutation lack immunoreactive PrA (ref. 18, Fig. 4), and that the PrA structural gene complements the deficiency of PrA and other vacuolar enzyme activities in *pep4* cells, the simplest hypothesis is that the *PEP4* gene encodes PrA. This hypothesis is also consistent with previously observed gene dosage effects of *PEP4* on PrA activity (7). We have recently found that the PrA structural gene carried on a single-copy centromere-containing plasmid complements the *pep4-3* mutation (12). Furthermore, a PrA structural gene deletion does not complement a *pep4-3* mutation in diploids, and this deletion maps genetically to the *PEP4* locus (12). Thus, it seems likely that PrA either processes vacuolar precursors or is required for the activation of a processing protease.

Note Added in Proof. After treatment of the intracellular mature and secreted forms of immunoprecipitated PrA with endoglycosidase F, the two polypeptides do not comigrate. This suggests that intracellular and secreted PrA probably differ by a non-carbohydrate modification.

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