Abstract. The vacuolar protease B of Saccharomyces cerevisiae is a subtilisin-like protease encoded by the PRB1 gene. Antibodies raised against a synthetic peptide and an Escherichia coli-derived PRB1 open reading frame (ORF) protein cross-react with authentic protease B from yeast. By using these antibodies, the posttranslational biosynthetic pathway of protease B has been elucidated. Preproprotease B is a 76-kD unglycosylated precursor that enters the endoplasmic reticulum (ER), where it receives one asparagine-linked (Asn-linked) and an undetermined number of non-Asn-linked carbohydrate side chains. The large glycosylated intermediate is proteolytically processed to a 39-kD form before exiting the ER. In the Golgi complex, the 39-kD form becomes 40 kD, due to elaboration of the Asn-linked side chain. The carboxy-terminal end of the 40-kD proprotease B undergoes protease A-mediated processing to a 37-kD intermediate, which in turn is quickly processed to 31-kD mature protease B. The ultimate processing step removes a peptide containing the Asn-linked chain; mature PrB has only non-Asn-linked carbohydrate.

The lysosome-like vacuole of the yeast Saccharomyces cerevisiae contains several hydrolytic activities, including protease A (PrA), protease B (PrB), carboxypeptidase Y (CpY), a 600-kD aminopeptidase, a repressible alkaline phosphatase, and at least one RNase (39, 65). In all cases studied to date, these hydrolases are synthesized as inactive glycoprotein precursors (reviewed in reference 28) that require the PEP4 gene product, protease A, for activation (2, 28, 66). Vacuolar hydrolases may be thought of as internally secreted proteins, as they traverse the ER and Golgi apparatus en route to the vacuole. Mutations that block transport from the ER or Golgi apparatus affect internally and externally secreted proteins equally (31, 57).

Some preliminary work on the structure of PrB and its precursors has been reported. In kinetic experiments using a 20-min pulse of [35S]methionine, Mechler et al. (43) identified a 42-kD precursor of PrB that is subsequently processed to the mature form. The relative molecular mass of the precursor was 3 kD smaller in tunicamycin-treated cells, but the relative molecular mass of the mature protease was unaffected (43). This kinetic precursor had the same relative molecular mass as the zymogen form of protease B observed to accumulate in a pep4 mutant (44). Mature protease B has been reported to be a glycoprotein that contains "no more than 0.5%" carbohydrate (61). A different group reported PrB to be a 33-kD glycoprotein with 8–9% neutral sugars and 1.5% amino sugars (32). The neutral/amino sugar ratio was suggestive of an Asn-linked carbohydrate moiety.

We recently reported the cloning and sequencing of PRB1 and also the purification and amino-terminal sequence of PrB (46, 47). Unexpectedly, analysis of the PRB1 DNA sequence showed an open reading frame (ORF) of 635 codons that would give rise to a primary translation product with a predicted (unglycosylated) Mr of 69.6 kD (47). The portion of the deduced amino acid sequence encoding mature PrB showed significant sequence similarity with members of the subtilisin family of proteases. Comparison of the deduced amino acid sequence and the amino-terminal protein sequence with the reported relative molecular mass of the mature and zymogen forms of PrB, as well as the sequences of the highly conserved subtilisins, led us to propose a model for the processing pathway for PrB (47). The proposed model stated that the entire ORF was translated and the resulting polypeptide received one or two Asn-linked and an undetermined number of non-Asn-linked side chains for a final molecular mass of ~75 kD. A protease other than PrA was responsible for cleaving 75-kD proPrB to generate a 44-kD protein that is identical to the zymogen form and has the same amino terminus as mature PrB. In the vacuole, PrA was responsible for a carboxy-terminal cleavage that generated mature PrB. Support for this model was presented by us at the June, 1987 Yeast Genetics and Molecular Biology meeting, and by others (42).

In this paper, we describe the isolation of antibodies that react with PrB and the use of these antibodies to elucidate...
posed processing model (47) has been essentially validated, and additional unanticipated details were discovered. At least three proteolytic processing steps are required for PrB maturation. The precursors have both Asn-linked and non-Asn-linked carbohydrate moieties, but mature PrB has only non-Asn-linked carbohydrate. Kinetically, preproprotease B appears to be posttranslationally rather than cotranslationally glycosylated. The processing step that generates the mature amino terminus of PrB occurs in the ER. The structures of some of the intermediates in the PrB processing pathway raise important questions about secretory protein localization and processing.

Materials and Methods

Chemicals and Media

Azocoll, Hide Powder Azure, keyhole limpet hemocyanin (KLH), and iso-
Strains, Genetic Methods, and Plasmids

Relevant strains and their genotypes are presented in Table I. The isolation of prb and pep mutants not in Table I has been described (26, 69). The procedures used for routine sporulation, dissection, and scoring of nutritional markers have been described (20). Scoring of prb mutations using Hidex Powder Azure overlays (46, 69) and pep mutations using the APE test have also been described (26, 66). The moeAp marker was scored with the Alcian blue test (16) on unheated cells (4). The olig and glf markers were scored by their effects on the relative mobility of CPr in a 7.5% polyacrylamide–SDS slab gel (29, 36). The relative mobility of CPr was determined on an immunoblot of the gel. The kex mutations were scored essentially as described (64). The sec88 marker was scored initially by inability to grow on yeast extract peptone dextrose (YPEP) at 37°C. This assignment was confirmed by measuring the relative molecular mass of immunoprecipitated radiolabeled CPr that had been synthesized under nonpermissive conditions in sec88 and sec18 cultures. Fluorograms of polyacrylamide–SDS gels were used to measure relative molecular mass. Bacteria were transformed by the CaCl2 method (41). Yeast were transformed by the LiAc method (24) with previously described modifications (66). Plasmid pUC19 is an E. coli expression vector (67). Plasmid YCp50 is a low copy, centromere-containing, yeast vector (34). Plasmid pCM5HH is a Ycpl50 derivative containing the PRB1 gene (46).

Amino-terminal Peptide

A synthetic pentadecamer peptide corresponding to the 14-amino-terminal amino acids of mature PrB (47) plus a cysteine (sequence = EFDTQN- waled) freshly DMSO-equilibrated Affi-gel 10 resin. After conjugation and turer's instructions. Approximately 40 mg of HPLC-purified genuine and Purified peptide was conjugated to Affi-gel 10 according to the manufac-
tional cross-linking reagent MBS is first reacted with the primary amine groups on a carrier molecule (KLH) and then cross-linked to a free sulf-
tations contained 0.2 nag of conjugate. The initial subcutaneous injection contained antibodies that recognized PrB on Western blots (7) at dilutions of 1:5000; we were unable to raise antibodies to proteinase K. (We suspected that the major extracellular protease of Neurospora crassa [1] also might be similar to protease B [47], so immunoblots of yeast extracts were probed with antibodies to the Neurospora protease. These antibodies also failed to cross-react with PrB.)

Three rabbits (R31, R32, and R35) were immunized with the first peptide–KLH conjugate, and three rabbits (R34, R36, and R37) were im-
munized with the second. All six rabbits were treated similarly. All injec-
tions contained 0.2 mg of conjugate. The initial subcutaneous injection contained antigen in 0.7 ml of 50% Freund's complete adjuvant and all sub-
sequent intramuscular injections contained antigen in 0.25 ml of 50% Freund's incomplete. The intramuscular injections were given 2 wk after the initial subcutaneous one, and then every 4–6 wk thereafter. The rabbits were bled once a week during the 3-wk period after the second and all subsequent intramuscular injections. The immune serum from R31, R32, and R35 contained antibodies that recognized PrB on Western blots (7) at dilutions of 1:100 to 1:250, from R37 at 1:32; an insignificant amount of antibodies that recognized PrB were obtained from R34 and R36. For most peptide antibody experiments, serum from R35 was used.

Three additional rabbits (R48, R50, and R52) were immunized under contract with Bethyl Labs (Montgomery, TX). For these three rabbits, all injections were subcutaneous with Freund's complete adjuvant. For rabbits R48 and R50 each injection contained 300 #g of protein (estimated from Coomassie Blue–stained polyacrylamide gels) from the insoluble fraction of the PrB ORF protein preparation. After seven injections, neither R48 nor R50 produced a reasonable titer of antibodies that recognized PrB. For rabbit R52 each injection contained 75–100 mg of polyacrylamide gel–purified protein from the insoluble fraction of the PrB ORF protein preparation. For all subsequent injections the protein was electroeluted (23; Elutrap; Schleicher & Schuell, Inc.) from the gel. After the fifth injection, R52 produced an excellent titer of antibodies that recognize protease B.

Purification of Antipeptide Antibodies

All steps of this purification were carried out at 4°C. Rabbit serum that reacted with PrB from yeast extracts was pooled and (NH4)2SO4 was added to a final 50% saturation. The 50% (NH4)2SO4 pellet was resuspended in, and dialyzed extensively against, 20 mM Tris, pH 7.2. After dialysis, insoluble material was removed by centrifugation at 12,000 g for 20 min. Ali-
quot of dialysate were loaded on the Affi-gel 10–peptide column, and the
flow-through was saved. The column was washed until the A$_{280}$ was <0.02, at which time antipeptide antibody was eluted with 0.2 M glycine, pH 2.3. Elution continued until the A$_{280}$ was <0.02. Eluted fractions were neutralized with 1 M Tris base, and the A$_{280}$ monitored. Peak fractions were precipitated in 2 M (NH$_4$)$_2$SO$_4$. The pellet was resuspended in and dialyzed against PBS. The breakthrough fractions were pooled and reapplied to the column until there was no longer a significant peak eluted. This column yielded 1-4 mg of antibody per elution.

**Antibodies to Protease B**

Because purified protease B was difficult to obtain, synthesis of a PrB peptide was commissioned and the peptide was used to generate and affinity-purified antibodies. The affinity-purification was successful at removing contaminating antibodies to many other proteins, but resulted in enrichment for antibodies to a 55-kD protein that is not encoded by the PRB locus even more than for antibodies to PrB (45; data not shown). The affinity-purified antibodies worked well on immunoblots, but were not very effective in immunoprecipitations. Interestingly, these antibodies showed a weak reaction against the Neurospora extracellular protease, and a strong reaction against protease K (data not shown). The reaction against protease K is not surprising insofar as eight residues of the peptide are identical to the amino terminus of the protease; the sequence of the Neurospora protease is not known.

The PRB ORF was placed under lac control in the plasmid pUCPRB1AH. The plasmid was constructed so that the lac promoter, operator, and ribosome-binding site directed the synthesis of the antibody peptide that consisted of the lacZ initiating Met, followed by 8-amino acid residues (ITPTLQAK), and then the entire 635-residue PRB ORF. Expression of a 77-kD protein was induced with the gratuitous inducer IPTG. The PrB peptide antibodies were used to verify that this 77-kD protein was encoded by the 644-codon chimeric ORF in the plasmid, and that the induced protein partitioned into an insoluble protein fraction (45; data not shown). Both the crude insoluble protein fraction and polycrylamide gel-purified ORF protein were injected into rabbits. The crude insoluble fraction was not an effective antigen, but the purified protein was. Antiserum to the gel-purified protein reacted strongly with PrB on immunoblots at dilutions of at least 1:500. This antiserum was also very effective in immunoprecipitation experiments. Unfortunately, the gel-purified 77-kD ORF protein could not be conjugated to Affigel 10 or Affigel 15 under any of several conditions recommended by the manufacturer. Therefore, for most experiments, the affinity-purified peptide antibody was used in immunoblots, and the 77-kD ORF protein antibody was used in immunoprecipitations.

**Immunoblots**

Yeast extracts were prepared from stationary phase, liquid YEPD-grown cultures. Approximately 3 x 10$^8$ cells (A$_{600}$ = 30) were harvested per culture in 13 x 100 mm glass tubes and washed once with 2 ml of phosphate extract buffer (20 mM sodium phosphate, pH 7.4, 0.15 M NaCl). To the cell pellets were added 0.4 g glass beads (0.44-0.46 mm diameter) and 50 #l 1% Triton X-100. The cell debris was removed by centrifugation at 27,000 g for 20 min. The proteins were separated by subjecting them to amide-SDS gels, 1.5 mm thick (36). For each gel, the samples were normalized so as to load comparable amounts of radioactivity per lane. The radioactive proteins were visualized by fluorography using either ENHANCE or AUTOFLUOR according to the manufacturers’ instructions. Addition of the AUTOFLUOR of gels to a final concentration of 5% considerably improved the handling and drying qualities of the gels. Either Kodak XAR or XRP film was used. As $^{35}$S decay does not produce enough energy to expose both sides of two-sided film, the emulsion on the exposed side of (dry) developed films was removed with household bleach. This reduced the background by 50% without affecting the signal.

**Results**

**Protease B in Crosses of a pep4 Mutant with alg5 and alg6 Mutants**

To determine the number of chains and the extent of glycosylation in mature PrB and its zymogen that accumulates in pep4 mutants, several known glycosylation mutations (alg5, alg6, glsl, and mnnl) were tested for their effects on PrB in both Pep4 and pep4 genetic backgrounds. Mutations in the ALG5 or ALG6 genes result in failure to add three glucose residues to the dolichol-linked glycolipid donor, which in turn reduces the efficiency of transfer of full-length chains to asparagine residues in polypeptides (22, 38, 54) including PrB (29). An alg5 and an alg6 mutant were each crossed to a pep4 mutant (BJ2589 x BJ3496 and BJ2590 x BJ3493, respectively). Protein transfer blots of extracts from the parent strains and several tetrads from these crosses were probed with antibodies to the PrB peptide. The alg6 Pep4 strains accumulated normal mature PrB, but the alg6 pep4 strains accumulated two protease B species (Fig. 1). One species comigrated with the pep4 zymogen form, and the other was smaller by 2.8 kD, or the size of one Asn-linked carbohydrate. These results indicate that the 40-kD zymogen form contains one Asn-linked carbohydrate side chain but that the mature enzyme contains no Asn-linked chains. The alg5 mutation had no effect on PrB, regardless of the Pep4 genotype (data not shown); this finding is in agreement with the relatively weak effect of the alg5 mutation on CprY (29) and secreted invertase (22).
PrB in gls1 and pep4 gls1 Mutants

After transfer of a completed GlcManGlcNAc2 chain from the dolichol carrier to an asparagine residue, the three glucose residues are removed (38) by the GLS1 gene product (12). A gls1 mutant was crossed to a pep4 mutant (BJ3622 × BJ3496). Protein extracts were made from the parent strains and several tetrads from this cross. Immunoblots of these extracts showed that the gls1 mutation had no effect on mature PrB. However, the PrB species that accumulated in the gls1 pep4 strain was larger than that in the GLS1 pep4 strain by 500-700 D, or the size of three to four glucose residues (Fig. 2). Therefore, the results with the gls1 and gls1 mutants were consistent with the PrB zymogen having one Asn-linked side chain, and the mature protease having none.

PrB in mnnl and pep4 mnnl Mutants

The mnnl mutant is reported to be defective in the addition of mannose residues to glycoproteins via α1-3 linkages and, therefore, defective in both Asn-linked and hydroxyly-linked glycosylation (8, 49, 52). An mnnl mutant was mated with a pep4 mutant (BJ3638 × BJ3493). Immunoblots of extracts from the parent strains and several tetrads from this cross were probed with antibodies to the PrB peptide. The mnnl mutation had no effect on PrB regardless of the PEP4 genotype (data not shown).

Determination of PrB Structure in sec61 and sec62 Mutants

Existence of the 635 residue ORF led us to believe that a precursor much larger than 42 kD should exist. The expected size of this precursor was >69 kD (47). When placed under restrictive conditions, sec61 and sec62 mutants accumulate unglycosylated precursor forms of CpY, mating factor α, and secreted invertase that fail to enter the ER (10). A larger PrB precursor would also be expected to accumulate in sec61 and sec62 mutants. Under nonpermissive conditions, these two mutants accumulated a 74-78-kD PrB species, 76-kD in subsequent discussion (see Figs. 8 and 9; these figures are fully discussed later). The observed size was close to that predicted from the PRB1 ORF. Tunicamycin had no effect on the size of the 76-kD form of PrB, which is in keeping with this being an unglycosylated, presecretory, or pre-ER, PrB species (45; data not shown).

Determination of PrB Structure in Mutants Defective in ER Functions

The sec18 mutation belongs to a group of conditional mutations that prevent transfer of secretory proteins from the ER to the Golgi apparatus under nonpermissive conditions. When placed at a nonpermissive temperature, sec18 mutants accumulate an under-glycosylated ER form of CpY and secreted invertase (13, 51). In the ER, the GLS1 gene product removes three glucose residues from each Asn-linked carbohydrate side chain. In gls1 mutants, the glucose residues are not removed, but this does not interfere with secretion and processing of the glycoprotein (12). These two ER-function mutations were tested for their effects on PrB in in vivo–labeled cells. (Under the labeling conditions used for this experiment, i.e., 10 min of labeling at 37°C followed by no chase, PEP4 strains behaved like leaky pep4 phenocopies. In other words, the PEP4 strains accumulated significant amounts of proprotease B.) Consistent with the immunoblot experiments described above, proprotease B was slightly larger in the gls1 strains than in the equivalent GLS1 strains.

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Figure 3. Some ER forms of PrB. Cultures were pregrown at 23°C in Wickerham minimal proline, preincubated for 10 min at 37°C, and labeled for 10 min at 37°C with [35S]sulfate without any subsequent chase. Under these conditions, PEP4 strains were slightly leaky pep4 phenocopies; that is, they accumulated a substantial amount of proPrB. Strains used and relevant mutations were: lane 1, BJ3658 (glsl); lane 2, BJ1984 (pep4); lane 3, BJ1983 (+); lane 4, BJ3655b (sec18); lane 5, BJ3656 (sec18 glsl).

(Fig. 3). Somewhat surprising was the finding that PrB in a sec18 mutant was nearly the same size as in a pep4 mutant (39 and 40 kD, respectively). This suggested that a major proteolytic processing event occurred before the protein left the ER. Except for the signal peptidase cleavage, we know of no other example of proteolytic processing in the ER of yeast. The relative molecular mass of PrB was smallest in the sec18 mutant and was incrementally larger in the pep4, sec18 glsl, and (by inference) pep4 glsl mutants (Fig. 3). Because a change in the GLS1 genotype results in a change in the size of PrB by three hexose units in these pairs, it can be inferred from Fig. 3 that the pep4 PrB species could be only one or two hexose residues larger than the sec18 PrB species.

To distinguish whether the differences in PrB relative molecular mass observed in pep4, sec18, and pep4 glsl mutants were due to elaboration of Asn-linked glycosylation, or some other event such as elaboration of hydroxyl-linked glycosylation, these mutants were labeled in the presence of tunicamycin. The PrB species that accumulated in the tunicamycin-treated pep4, sec18, and pep4 glsl mutants were indistinguishable from one another (Fig. 4). Therefore, the relative molecular mass difference between the PrB species in a pep4 and a sec18 mutant must be due entirely to a difference in the Asn-linked side chains. Insofar as the Asn-linked side chains on invertase that accumulated in the sec18 mutant were of the composition ManαGlcNAc2 (12), the side chain on protease B in the pep4 mutant was probably of the composition ManαGlcNAc2. The data in Fig. 4 also confirm that mature PrB does not contain any tunicamycin-sensitive carbohydrate.

Strains carrying mutations in the SEC11, SEC53, or SEC59 genes, whose gene products are believed to be required for functions between those of the SEC61/SEC62 pair and SEC18, were examined for their effects on PrB processing. The SEC11 gene is believed to encode a subunit of yeast signal peptidase (6). After in vivo labeling under nonpermissive conditions and immunoprecipitation of protein extracts, the PrB species that accumulated in a sec11 mutant was the same size as that in a sec18 mutant (Fig. 5). Therefore, the SEC11 gene product is not required for processing of PrB from 76 to 39 kD.

Mutants carrying alleles in either SEC53 or SEC59 genes are known to accumulate early ER forms of CpY and secreted invertase under nonpermissive conditions (15). Both the sec53 and sec59 mutants gave identical results with respect to PrB (Fig. 6): two proteins accumulate that cross-react with antibodies to the 77-kD ORF protein. Protease B is the same size as mature PrB (31 kD), and the other is about the same size as unglycosylated proPrB (37 kD). These results are preliminary: it is not known from what part(s) of the PRB1 ORF these two proteins come, nor is it known whether either of them is glycosylated.
Figure 6. PrB-related protein species accumulating in a sec59 mutant. The sec59 strain was grown at 23°C in Wickerham minimal proline medium, preincubated for 10 min at 37°C, and labeled for 10 min at 37°C with [³⁵S]sulfate without any subsequent chase. The SEC59 strain was grown at 23°C in Wickerham minimal proline medium and labeled for 10 min at 23°C with [³⁵S]sulfate followed by a 40-min chase. Cell extracts were subjected to immunoprecipitation with antibodies to the 77-kD ORF protein. The sec59 mutant accumulated two forms of PrB, one that comigrated with mature PrB, and another that was 37-38 kD, or in the size range of the PrBzymogen without its Asn-linked side chain. The sec53 (BJ2388) and sec59 mutants gave identical results (not shown). Since antibodies to the 77-kD ORF protein were used, we do not know from which part of the open reading frame either of these proteins were derived. Strains used and relevant mutations were: BJ2390 (see59) and BJ1983 (SEC59).

Figure 5. Accumulation of a 39-kD species of PrB antigen in a sec11 mutant. Cultures were grown at 23°C in Wickerham minimal proline medium, preincubated for 10 min at 37°C, and labeled for 10 min at 37°C with [³⁵S]sulfate without any subsequent chase. Protein extracts were subjected to immunoprecipitation with antibodies to the 77-kD ORF protein. Both the see11 and see18 mutants accumulated a 39-kD form of PrB-related protein. Strains used and relevant mutations were: BJ3748 (sec11) and BJ4378 (see11).

Determination of PrB Structure in Mutants Defective in Golgi Complex Functions

The sec7 and sec14 mutations belong to a class of mutations that are conditionally defective in translocation from the Golgi complex. When placed at a nonpermissive temperature, the sec7 mutant is known to accumulate a slightly underglycosylated form of proCpY and secreted invertase. Under these same conditions the sec14 mutant accumulates proCpY that is identical in size to that found in pep4 mutants (57). We confirmed that the sec7 (BJ2159) and sec14 (BJ2160) mutations had a similar effect on PrB processing as they did on CpY. The sec14 and pep4 mutants accumulate identical-sized PrB species, and the sec7 mutant accumulated a PrB species of a size intermediate to that of the sec18 and sec414 mutants (data not shown).

Short Lifetime of the 76-kD Precursor

While the 40-kD precursor was previously identified in kinetic experiments (44), the 76-kD precursor had not been identified, even in the absence of a chase. This suggested that the 76-kD precursor is rapidly processed to the 40-kD intermediate, and, that after a 20-min pulse (44), the fraction of labeled PrB represented by the 76-kD species must be almost zero. This hypothesis was borne out by a pulse-labeling experiment in which [³⁵S]sulfate was added to one culture, and samples were removed at several time points (Fig. 7). There was no chase; at the end of the labeling interval the samples were stopped with cold TCA. After 4 min of labeling at 18–21°C (Fig. 7, lane 4), the 76-kD species already accounted for less than half of the total labeled PrB. After 6 min of labeling (Fig. 7, lane 5), the fraction of labeled PrB represented by the 76-kD species was nearly zero. The 76-kD species was processed to the 40-kD intermediate in one quick step, with no trace of the other half of the precursor (Fig. 7). (The antiserum used in this experiment does recogno
**Figure 7.** PrB precursors seen with increasing length of pulse labeling. Cultures were grown in Wickerham minimal proline medium, starved for sulfur, and labeled with [³⁵S]sulfate. Cell extracts were subjected to immunoprecipitation with antibodies to the 77-kD ORF protein. Lanes 2–9 represent a pep⁴ mutant (BJ1984) labeled for 1:12, 2:07, 4:05, 6:01, 7:05, 10:05, 14:22, and 20:10 min/label, respectively, with no chase. Lane 1, BJ3947 (sec⁶); lane 10, BJ3748 (sec⁸); lane 11, BJ1983 (+); lane 12, BJ1984 (pep⁴); lane 13, BJ3044 (prbl-Δ1.6). For lanes 11–13, the cultures were labeled for 15 min, and chased for 40 min. All samples were adjusted to a comparable number of cpm before loading.

**Processing of PrB in Pulse-Chase Experiments**

Labeling with sulfate for <4 min resulted in unsatisfactory level of label incorporation, yet after labeling for 4 min, the early form(s) of labeled PrB were difficult to see because they represented a very small fraction of the total labeled PrB. Accordingly, [³⁵S]methionine (Tran³⁵S) labeling was used. A preliminary experiment with a 30–40-s pulse of label provided excellent resolution of early species (Fig. 8).

**Figure 8.** PrB in a pulse–chase experiment. This figure shows the results of two [³⁵S]methionine pulse–chase labelings of strain BJ1983 (MATc irpl). Cells were grown in Wickerham minimal proline medium supplemented with adenine, histidine, leucine, lysine, tryptophan, and uracil. Enough cells were harvested to give 1.5 OD per time-point and resuspended at 4.5 OD per ml in fresh medium plus 0.55 mCi [³⁵S]methionine per time-point at 25°C. In both cultures, the radioactive label was chased by adding unlabeled methionine and cysteine to a final concentration of 3.6 mM. For both cultures, the chase was initiated 30–40 s after adding label and samples were taken after initiating the chase at the times indicated. Control strains + (BJ1983 with a 40-min chase), pep⁴ (BJ1984), sec⁸ (BJ3748), and sec⁶ (BJ3947) were labeled with [³⁵S]sulfate. Molecular mass standards are shown on the right side of the figure. All samples were adjusted to a comparable number of cpm before loading.
Figure 9. PrB and CpY in a pulse–chase experiment. The results of three [35S]methionine pulse–chase labelings of strain BJ3044 (prbl-Δ1.6) transformed with either the control vector YCp50 (C) or the PRBI-containing vector pCM5HH (A and B) are shown. Cells were grown in Wickerham minimal proline medium supplemented with adenine, histidine, leucine, lysine, tryptophan, alanine, and glycine. (Extra nutritional supplements allowed the cells to grow and label better without interfering with PRBI expression.) Enough cells were harvested to give 2 OD per time-point, and resuspended at 8 OD per ml in fresh medium. The three cultures were grown at 25°C with (A) or without (B and C) tunicamycin for 15 min before adding [35S]methionine (2.77 mCi for A and C, 4.42 mCi for B). In each culture, the radioactive label was chased by adding an equal volume of medium containing 33 mM each of unlabeled methionine and cysteine. For A (Prb+, +tunicamycin), the chase was initiated 1:58 min/s after adding label. The time-points were taken at 1:30, 3:30, 6:15, 12, and 24 min/s after adding label (A, lanes 1-5, respectively). For B (Prb+, -tunicamycin), the chase was initiated at 1:20 min/s after adding label. Samples were taken at 0:40, 2:20, 3:30, 6:00, 12, 24, 48, and 92 min/s after adding label (B, lanes 1-8, respectively). For C (Prb-, -tunicamycin), the chase was initiated at 1:30 min/s, and samples were taken at 0:40, 2:30, 3:30, 6:00, and 12 min/s after adding label (C, lanes 1-5, respectively). For D-F, the cell extracts used for A-C were subjected to a second immunoprecipitation with anti-CpY (see Materials and Methods). Control strains BJ3748 (sec18), BJ1984 (pep4), BJ3947 (sec61), and BJ3944 (sec62) were labeled with [35S]sulfate. All samples were adjusted to a comparable number of cpm before loading. Molecular mass standards are indicated on the left side of the figure. On the right side, major forms of PrB or CpY are indicated by their molecular mass or by the nomenclature of Stevens et al. (57).

That the initial species detected with no chase matures into a slightly larger species by 1 min of chase is evident. However, a 30-40-s pulse still provided an unsatisfactory level of label incorporation and also revealed extraneous bands >76 kD that may or may not be related to PrB. In a subsequent set of pulse–chase experiments, these problems were addressed by: (a) taking the first time point at 40 s, but continuing the labeling pulse up to 80 s (Fig. 9); and (b) labeling a prbl-Δ1.6 strain that was transformed with the control vector YCp50 (Fig. 9 C), and the same strain transformed with...
a PRBl-containing YCp50 derivative, pCM5HH (Fig. 9 b). Protease B was first synthesized as a 76-kD unglycosylated precursor that comigrated with the sec61/sec62 species (Fig. 9 B). This precursor chased into a short-lived, larger intermediate of \( \sim 78 \) kD (Fig. 9 B, lane 2). The increase in size appeared to be due to Asn-linked glycosylation, as the increase was not seen in tunicamycin-treated cells (Fig. 9 B). Within 3:30 min of adding label (Fig. 9 B, lane 3), nearly all of the 78-kD intermediate was processed, in one step, to a 39-kD species that comigrated with the form found in sec18 mutants. By 24 min (Fig. 9 B, lane 6), all of the PrB had chased to the 40-kD intermediate that accumulates in pep4 mutants. (In the experiment with a 30-s pulse, a discrete form, intermediate in size between the sec18 and pep4 forms, of PrB could be identified with shorter exposure times than that shown.) At 24 min, a slightly smaller, 37-38-kD intermediate appeared, which in turn was chased to the 31-kD mature PrB. The size of the 37-38-kD intermediate is reduced in the presence of tunicamycin, indicating that it is glycosylated (Fig. 9 A). The labeled cell extracts that gave rise to Fig. 9, A–C were subsequently subjected to another immunoprecipitation with antibodies to CpY (Fig. 9, D–F). At 25°C, processing from the ER form to the pep4 zymogen form and then to the mature form was similar for PrB and CpY.

**Amino Terminus of the 40-kD Species**

In an earlier report, we predicted that protease A-mediated processing of the 40-kD intermediate to 31-kD mature PrB occurred via a carboxy-terminal cleavage (47). Proof of this hypothesis would come from an unequivocal determination that the two species had different carboxy termini but identical amino termini. Demonstration of the former would have been technically much more difficult, so we pursued the latter. Unfortunately, so far we have been unable to purify enough of the 40-kD precursor for sequencing, but the peptide antibody allowed a demonstration that both the 40-kD precursor and mature protease B probably share the same amino terminus. A PEP4 and a pep4 strain were labeled with \([^{35}S]\)sulfate, chased, and protein was extracted. One-half of each extract was subjected to two sequential immunoprecipitations with antibodies to the PrB peptide, and the other half was treated with pronase, and the remaining one-fourth was subjected to another immunoprecipitation with antibodies to CpY.

**Figure 10.** (A) Shared amino terminus of the 31-kD mature and 40-kD zymogen forms of PrB. Radiolabeled cell extracts of BJ1983 (PEP4, lanes 1 and 2) and BJ1984 (pep4, lanes 3 and 4) were prepared as before. PrB was immunoprecipitated with either affinity-purified antibodies to the peptide-KLH conjugate (lanes 1 and 3), or antiserum to the 77-kD ORF protein (lanes 2 and 4). The affinity-purified antibodies identified an additional 16-kD protein common to both strains. See text for further details. (B) Three hypothetical interpretations for the results in A. The figure is discussed fully in the text. Definition of symbols used: AA, amino acid followed by position number with respect to the amino terminus of mature PrB; N and C, amino and carboxyl termini of the polypeptide, respectively; t or ~, proposed site of cleavage(s) necessary to generate the 16-kD degradation product. The horizontal lines between N and C, represent the polypeptide drawn to a scale based on the apparent molecular mass of the glycoprotein. The small open box designates the target site for the affinity-purified antibodies. The upside-down Y represents the hypothetical location of the Asn-linked carbohyd late chain of the PrB zymogen.
addition, there was one major PrB degradation product of 16 kD common to both strains. In both was subjected to two sequential immunoprecipitations with anti-serum to the 77-kD ORF protein. The bands indicated on this immunoblot are A, 40-kD pep4 zymogen form of PrB; B, 37-kD prb1-628 inactive form; C, 31-kD PEP4 PRB1 mature form. The strains used in this immunoblot were: BJ3496 (MATa PRB1 pep4::HIS3), BJ52 (MATa prb1-628 PEP4), one parental dip-type tetrad and one tetraphyle tetrad from this cross.

was subjected to two sequential immunoprecipitations with antiserum to the 77-kD ORF protein. In both PEP4 and pep4 strains, the antibodies to the amino-terminal peptide recognized the expected 31- or 40-kD PrB peptide. The bands indicated on this immunoblot are A, 40-kD pep4 zymogen form of PrB; B, 37-kD prb1-628 inactive form; C, 31-kD PEP4 PRB1 mature form. The strains used in this immunoblot were: BJ3496 (MATa PRB1 pep4::HIS3), BJ52 (MATa prb1-628 PEP4), one parental di-type tetrad and one tetraphyle tetrad from this cross.

Figure 11. Epistasis of pep4 to prb1-628 with respect to PrB structure. All strains were grown to stationary phase in YEPD. Protein was extracted and fractionated on a polyacrylamide gel and transferred to nitrocellulose. The blots were incubated with affinity-purified antibodies to the amino-terminal PrB peptide. The bands indicated on this immunoblot are A, 40-kD pep4 zymogen form of PrB; B, 37-kD prb1-628 inactive form; C, 31-kD PEP4 PRB1 mature form. The strains used in this immunoblot were: BJ3496 (MATa PRB1 pep4::HIS3), BJ52 (MATa prb1-628 PEP4), one parental di-type tetrad and one tetraphyle tetrad from this cross.

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Protease B in prb2, prb3, and prb4 Mutants

In an attempt to identify a stable nonsense fragment of PrB, we screened immunoblots of protein extracts from our collection of 125 prb1 mutants with the affinity-purified antibodies to the PrB peptide; such a stable nonsense fragment could be used to demonstrate which end of PrB is processed by protease A (21, 68). None of the prb1 mutants synthesized a stable PrB nonsense fragment, but one of them, BJ52, did contain an interesting protease B species. This mutant accumulated some mature-size PrB as well as a 37-kD protein that cross-reacted with the antibodies to the PrB peptide (Fig. 11). When BJ52 was crossed to a pep4 mutant (BJ3496), it was observed that the pep4 mutation was epistatic to the prb1 mutation, because the 40-kD species was the only one present in the pep4 prb1 mutant (Fig. 11). The simplest explanation for these results is that there is a processing intermediate between the 40-kD zymogen and the 31-kD mature forms of PrB. The mutation in BJ52 does not interfere with the protease A catalyzed processing step, but reduces the rate of the subsequent one.

Novel PrB Processing Intermediate in a prb1 Mutant

In an attempt to identify a stable nonsense fragment of PrB, we screened immunoblots of protein extracts from our collection of 125 prb1 mutants with the affinity-purified antibodies to the PrB peptide; such a stable nonsense fragment could be used to demonstrate which end of PrB is processed by protease A (21, 68). None of the prb1 mutants synthesized a stable PrB nonsense fragment, but one of them, BJ52, did contain an interesting protease B species. This mutant accumulated some mature-size PrB as well as a 37-kD protein that cross-reacted with the antibodies to the PrB peptide (Fig. 11). When BJ52 was crossed to a pep4 mutant (BJ3496), it was observed that the pep4 mutation was epistatic to the prb1 mutation, because the 40-kD species was the only one present in the pep4 prb1 mutant (Fig. 11). The simplest explanation for these results is that there is a processing intermediate between the 40-kD zymogen and the 31-kD mature forms of PrB. The mutation in BJ52 does not interfere with the protease A catalyzed processing step, but reduces the rate of the subsequent one.

Protease B in prb2, prb3, and prb4 Mutants

The prb2-prb4 mutants were identified in this laboratory in the same mutageneses that generated the prb1 mutants (68). The prb2 and prb3 mutants were reported to be Prb+ on yeast extract peptone glycerol (YEPG), but Prb- on YEPD. The prb4 mutants were reported to take four times as long as PRB strains to develop a halo in the HPA test (68). All of the available mutants in these three complementation groups were tested by the immunoblot technique, using protein extracts from cells that had been allowed to grow to stationary phase in liquid YEPE medium. Of the two prb2 mutants, one grew too poorly to work with, and one proved to be a prb1 mutant. Most of the prb3 mutants accumulated a normal amount of mature PrB under these conditions, but three of them accumulated far less PrB than the PRB strain (BJ119, BJ121, and BJ126). Protein extracts from the three available prb4 mutants were also analyzed on immunoblots. All three were completely lacking in PrB protein. Therefore,
Figure 12. Summary of PrB maturation pathway. This figure is explained fully in the discussion. N and C represent the amino and carboxy termini of the primary translation product. The horizontal lines represent the polypeptide backbone drawn to scale. Open arrows signify an interorganelle translocation step; closed arrows signify an intraorganelle processing step. 

No suggestion of the total amount is implied. The inverted Y represents the core of one Asn-linked carbohydrate chain. The two circles attached to the Y represent elaborations of the core. The numbers to the right of the figure indicate the apparent molecular mass of each intermediate.

neither the PRB3 nor the PRB4 gene products appear to be necessary for proper PrB maturation.

Protease B in Immunoblots of Several pep Mutants

Strains bearing a mutation at the PEPI-PEP15 loci were tested for accumulation of PrB precursors. All of the pep mutants were previously identified as being deficient in the expression of CpY activity, and most pep mutants are also deficient in the expression of other vacuolar hydrolase activities (26). Several of these mutants accumulate an inactive precursor form of CpY, proCpY, but except for PEP4, the function of the PEP gene products is not well understood (unpublished results). Protein extracts from one member or more of each of the pep1 through pep15 complementation groups were probed with antipeptide antibodies on a Western blot. Most of these mutants accumulated the 40- and/or the 31-kD PrB species. In addition, four or five of these mutants, namely pep3,5,7,12, and maybe pep8, accumulated an additional PrB species of ~37 kD. In none of these mutants did the 37-kD species appear by itself; rather it was always accompanied by the 40- and/or 31-kD species (data not shown). Thus, analysis of these mutants has failed to surface defects in the activity responsible for generating the mature amino terminus of PrB.

Discussion

Summary of Proposed Protease B Posttranslational Biosynthetic Pathway

In a recent paper, we reported the PRBI DNA sequence, the protease B amino-terminal protein sequence, and a model for the processing pathway for PrB (47). The DNA sequence predicted a 635 codon ORF that would encode a 70-kD polypeptide with five potential sites for Asn-linked glycosylation. The mature protease was only 33 kD, however, and we knew that 280 codons preceded those that encoded the amino terminus of mature PrB. The only precursor to protease B that had been reported was a 42-kD kinetic precursor of identical relative molecular mass as a PrB species identified in a pep4 mutant (43, 44). This 42-kD species had at least one Asn-linked side chain as judged by tunicamycin treatment. The mature protease did not have any tunicamycin- or Endo H-sensitive carbohydrate moieties (43, 44, 47), although it was clearly a glycoprotein (32, 47, 61). Also, a review of the literature revealed that yeast proteins were preferentially glycosylated at the Asn-Xaa-Thr consensus rather than the Asn-Xaa-Ser consensus sequence for Asn-linked glycosylation (47). The PRBI ORF encoded one of the N-X-T and four of the N-X-S sequences; the one containing Thr was the most carboxy-terminal of the five. Finally, the sequence similarity with the other proteases showed that all of the others in this group ended within one or two amino acids of each other, but the otherwise very similar protease B ran on for another 50 or 60 amino acids before terminating (47). From all of these data, we built a model for the processing pathway of PrB (47). In this model, preproPrB is synthesized as a 69.6-kD polypeptide. In an unspecified order, the preproPrB is glycosylated and proteolytically cleaved to yield an inactive, 40–44-kD glycoprotein that has: the same amino terminus as mature PrB; as much as 2.9 kD of non-Asn–linked carbohydrate on the mature portion of the enzyme; and 3.0 kD of Asn-linked glycosylation attached to the Asn at position 314 (relative to the amino terminus of the mature protein). Thiszymogen form of PrB would be cleaved on the carboxy-terminal side, near to residue 239 where the homologous subtilisins are also terminated, by the PEP4 gene product, PrA (2, 66), to generate a 34-kD mature enzyme that contains 2.9 kD of non-Asn–linked carbohydrate.

Summary of the Actual Protease B Posttranslational Biosynthetic Pathway

We have attempted to dissect the posttranslational biosynthetic pathway for PrB; the results are summarized in Fig. 12. PrB traverses the ER and Golgi complex en route to its
final destination, the vacuole. The full-length 76-kD primary translation product, preproPrB, accumulates transiently before being glycosylated and proteolytically processed. The order of the intervening events in the PrB processing pathway is not entirely clear, but somehow the unglycosylated 76-kD form is transformed into the glycosylated 39-kD ER form. This ER form has both Asn-linked and non-Asn-linked (presumably hydroxyl-linked) carbohydrate, and probably has the same amino terminus as the mature PrB. The much smaller proPrB enters the Golgi complex where a small addition is made to the carbohydrate component. The fully glycosylated form of proPrB then undergoes two proteolytic cleavages in rapid succession, losing its Asn-linked side chain with the second of these clips, to give rise to the vacuolar mature PrB. Mature PrB contains no Asn-linked carbohydrate, but does contain an undetermined amount of carbohydrate, presumably hydroxyl linked. Whether or not a PrB signal peptide is removed is not clear. The exact fate of the 260–280-amino acid amino-terminal polypeptide is not yet clear, but it is possible that this polypeptide comigrates with proPrB (discussed below).

**PrB Processing Pathway from Translation through the Golgi Complex**

Whether the full-length of preproPrB enters the ER is not clear from the results of these studies. The results presented in Figs. 7 and 9 show that a 76-kD preproPrB accumulates at the nonpermissive temperature in sec61 and sec62 mutants, which are blocked at the cytoplasm–ER translocation step (10). A PrB precursor of the same relative molecular mass also accumulates transiently in pulse-labeled SEC strains (Figs. 7, 8, and 9). The size of the 76-kD species is not affected by tunicamycin, an inhibitor of Asn-linked glycosylation (35), in either sec or SEC mutants, and data not shown.

In pulse–chase experiments, the sequence of events after the appearance of the unglycosylated 76-kD species is not clear. In Fig. 8, PrB appears initially as a 76-kD protein that becomes slightly larger (2 kD?), and is then transformed into a glycosylated 39-kD protein without any smaller intermediates. From the data in Fig. 9 it appears that the 76-kD precursor does chase into a larger 78-kD form in the absence, but not the presence, of tunicamycin (cf. lanes 1–3 of Fig. 9, A and B), as if the full-length 76-kD primary translation product enters the ER and receives some amount of glycosylation (Asn linked at least) before being processed into a smaller (39-kD) intermediate. These data suggest then, that glycosylation is a posttranslational rather than cotranslational event for protease B. However, the apparent posttranslational glycosylation (and by implication, translocation) could be a reflection of the location of the acceptor Asn at residue 314, 41 residues shy of the carboxyl terminus, rather than a true indication of posttranslational translocation. If the distance between the inner surface of the ER membrane and the peptide site in a ribosome is ~70-amino acid residues, cotranslational translocation of the protein would still result in termination of translation before glycosylation simply because the distance between the acceptor and the end of the polypeptide is substantially <70 residues. That the unglycosylated 76-kD species detected in pulse–chase experiments is the same size as the species that accumulates outside the ER in the sec61 and sec62 mutants is unexpected, however, if signal peptides act before completion of translation. If the 76-kD precursor chases into a 78-kD intermediate, then all of the 76-kD preproprotease B must enter the ER.

One troublesome observation, however, is the 93-kD species seen in Fig. 9 B but not 9 C. In Fig. 9, B and C the exact same prbl Δ1.6 strain was transformed with either YCp50::PRB1 or YCp50, respectively; labeled in the same media on the same morning; extracted and immunoprecipitated at the same time; and fractionated on the same polyacrylamide gel.) Furthermore, the 93-kD protein could have one Asn-linked side chain; the size of this species appeared to be affected by tunicamycin (smaller by ~3 kD). One must consider the possibility that preproprotease B undergoes extensive hydroxyl-linked glycosylation, like the low density lipoprotein receptor of mammalian cells (9, 59), before being processed to the 39-kD form.

There are three objections to the hypothesis that protease B has a 93-kD intermediate, however. First and foremost, the kinetics of appearance and disappearance for this putative intermediate do not look quite right: it appears and disappears a little too late to be an intermediate between the 76- and 39-kD forms. Second, insofar as hydroxyl-linked glycosyl moieties in the ER of yeast are thought to consist of Man, units (18), ~100 serine or threonine residues would have to be glycosylated to account for an additional M, of 16 kD. Even taking into account the aberrant migration of glycoproteins in polyacrylamide gels, wherein these proteins appear larger than they actually are (56), most or all of the 83 serine plus threonine residues of preproprotease B would have to be glycosylated with Man in order to account for this increase in size. Third, a 93-kD band appears in the prbl Δ1.6 lane of Fig. 7 (lane 13). Fig. 7 does not provide a very good control for Fig. 9, but still, it does weaken the possibility that the 93-kD protein is a PrB intermediate.

The relative molecular mass of the primary translation product (76 kD) is somewhat larger than predicted from the deduced amino acid sequence (70 kD; 47). This aberrant migration is also observed with the IPTG-induced ORF protein (77 kD observed vs. 71 kD predicted). This anomaly may result from the net charge of +12 on this protein, of which +11 is found in the first 280 amino acids. This high net charge and high charge density (111 of 280 residues) also makes us wonder whether this part of the protein passes through a membrane at all.

No species intermediate in size between 76 and 39 kD was seen. The results with mutants having conditional ER defects combined with the pulse–chase experiments in Figs. 7, 8, and 9, show that the 39-kD PrB species appears very early and is in the ER. Four secretory mutants that have conditional defects in ER functions, namely sec18, sec11, sec53, and sec59, were tested under nonpermissive conditions to determine what form(s) of protease B antigens accumulated. The sec18 mutation confers a conditional defect in translocation of proteins from the ER to the Golgi complex; under nonpermissive conditions sec18 mutants accumulate an ER precursor form of CpY (known as "pl") and secreted invertase (13). The 39-kD kinetic intermediate comigrated with a PrB antigen found in a sec18 mutant under nonpermissive conditions (Figs. 7, 8, and 9), thereby showing this kinetic intermediate to be an ER species. The SEC11 gene product is believed to be part of a yeast signal peptidase complex. Signal peptidase
in eukaryotes is believed to consist of a multimeric complex (3, 14), and the SEClI sequence predicts a protein that is similar in size and pI to the smallest of the subunits described by Baker and Lively (3, 6). Mutants lacking the SEClI gene product fail to cleave the signal peptide of CpY and secreted invertase, and have slowed kinetics of secretion for these two proteins (6). Other proteins are affected to different extents in the secIl mutant; some proteins are not affected at all, others are completely blocked in the ER, and still others are slowed in their kinetics of secretion (50). The secIl mutant accumulates a 39-kD form of PrB that comigrates with that found in the secll8 mutant (Fig. 5); we have not tried to assess the kinetics of PrB secretion in the seclll mutant. The SEC53 and SEC59 gene products at one time were believed to be necessary for entry into the ER, but now their function is unclear (15). The sec53 and sec59 mutants are conditionally defective in an unknown, early ER function and accumulate an aberrant, underglycosylated form of CpY and secreted invertase (15). Under nonpermissive conditions, both of these mutants accumulated two PrB-related proteins, one of which resembled mature PrB, and the other of which resembled underglycosylated proPrB (Fig. 6, sec53 not shown). Because the antibodies used in the sec53/sec59 experiments react with epitopes along the entire length of preproPrB, we do not know whether either of the two PrB species was derived from the amino-terminal half of the full-length protein. It seems possible that the smaller of these would be derived from the amino-terminal half, because one would not expect mature PrB to accumulate in mutants that are blocked at an ER step of secretion. In kinetic experiments, the 39-kD ER form of PrB chased to a 40-kD species (Figs. 7, 8, and 9). This 40-kD species comigrated with the PrB zymogen found in pep4 mutants (Figs. 7 and 9), as well as that found in a secll4 mutant (not shown). The pep4 mutation leads to an accumulation of inactive precursors for most soluble vacuolar hydrolases (21). The secll4 mutation confers a conditional defect in translocation of secretory proteins from the Golgi complex (13). Therefore, processing of the PrB zymogen to mature PrB occurs either in a late Golgi compartment or in the vacuole, as it does for CpY (13).

**Vacuolar Processing Steps in PrB Maturation**

In kinetic experiments, the 40-kD proPrB is processed into a 37-kD intermediate that retains an Asn-linked side chain, and subsequently into 31-kD mature PrB (Fig. 9, A and B). The PEP4 gene product, PrA (2, 66), is required for the 40 \(\rightarrow\) 37-kD processing step, as this step does not normally occur in pep4 mutants (e.g., Fig. 2). The gene products of several other PEP genes were required for one or both steps of vacuolar processing of PrB, but there is no evidence that any of these genes encodes a protease.

The short-lived 37-kD intermediate has probably been observed in other experiments as well. During an attempted purification of proPrB from a pep4 mutant, the 40-kD zymogen species started degrading into a 37-kD form and then rapidly degraded into mature PrB in vitro (45). The 37-kD kinetic intermediate is also approximately the same size as a PrB antigen found in several mutants. The prbl-628 mutant (BJ52) accumulated a 37-kD PrB protein that was not visible in prbl-628 pep4 mutants (Fig. 11). The epistasis of the pep4 mutation indicates that the prbl-628 allele encodes a PrB protein that cannot undergo or only slowly undergoes the 37 \(\rightarrow\) 31-kD processing step. Strains that are mutant in the pep3, pep5, pep7, pep12, and possibly pep8 loci also accumulated a 37-kD PrB species. It is possible that PrA is responsible for the final processing step and these other pep mutants accumulate the 37-kD PrB form as a consequence of their PrA deficiency, but it is also possible that the accumulation of the 37-kD species is a clue to some other physiological defect shared by this subset of pep mutants. This same subset of pep mutants has been reported to share other phenotypes not common to all pep mutants (27).

The relatively short-lived 37-kD intermediate is approximately the same size as the PrB zymogen found in tunicamycin-treated cells. This is simply a coincidence, because the size of the 37-kD kinetic intermediate was itself sensitive to tunicamycin treatment (Fig. 9). A 37-kD form of PrB also accumulated in the sec53 and sec59 mutants. Again, we do not think that this is the same as the 37-kD kinetic intermediate because these two sec mutants accumulate ER membranes and aberrant, underglycosylated ER forms of other proteins (13, 15).

**Identity of Proteases Involved in PrB Processing**

There are at least three proteolytic processing steps necessary for the maturation of protease B: 76 (78 kD) \(\rightarrow\) 39 kD, 40 \(\rightarrow\) 37 kD, and 37 \(\rightarrow\) 31 kD. (At present there is no clear evidence pertaining to a signal peptide cleavage event.) The penultimate processing step, conversion of 40 kD to 37 kD, must be mediated by the PEP4 gene product, PrA (2, 66), because it does not occur in pep4 mutants. That prcl mutants are PrB+ excludes CpY as the protease responsible for the final maturation event; there are no data on whether PrA, PrB, or another protease mediates this final step. The data exclude the SEC11 gene product (part of the signal peptidase complex; 6), the KEX1-encoded carboxypeptidase, and the KEX2-encoded endoprotease as being responsible for the initial 76 (78 kD) \(\rightarrow\) 39 kD cleavage. It is interesting to note that a two codon change at the amino terminus of mature PrB (Glu-Phe changed to Asp-Leu; data not shown) still results in prbl complementation. Presumably, the protease responsible for the cleavage in the ER has some flexibility in the target sequences whose cleavage it catalyzes.

**PrA Cleaves the Carboxy-terminal End of Proprotease B**

Based on discrepancies in predicted and observed molecular masses for proPrB and mature PrB, we postulated that the PEP4-mediated processing step occurs at the carboxy terminal of proPrB (47). Evidence supporting this hypothesis is presented in Fig. 10. Both proPrB and mature PrB share a common semistable degradation product of the same size that is recognized by antibodies against the amino terminus of mature PrB. This suggests that these two forms share a common amino terminus, and that the subsequent processing steps must occur at the carboxy end. Furthermore, the identification of a penultimate processing step that removes 3 kD but yields a product that contains an Asn-linked side chain requires that there be at least 18–20 amino acids distal to the one Asn-linked side chain on proPrB. This presents no problem for carboxy-terminal processing, as there are 41 amino acids distal to the potential glycosylation site. However, for
amino-terminal processing, the amino terminus of proPrB would have to be at, or upstream of, the Glu at position -86 relative to the amino terminus of mature PrB, since the potential tripeptide glycosylation acceptor is at position -68. If this were true, it would require that the 635-amino acid, 76-kD precursor consist of two parts: an amino-terminal half of 194 amino acids that accounted for 39 kD, and a carboxy-terminal half of 441 amino acids that only accounted for 37 kD (unglycosylated molecular masses). Clearly the data are in agreement with carboxy-terminal processing, and not with amino-terminal processing, by PrA.

**Fate of the Amino-terminal 280 Amino Acids of Preproprotease B**

The fate of the 280–amino acid amino-terminal polypeptide is unclear. The polypeptide was not apparent in the experiments represented by Figs. 3, 4, 5, and 7, where one would expect to see it. Part of the answer could be in Fig. 9. In Fig. 9 A, it appears that the tunicamycin treatment was somewhat ineffective, as a significant amount of 39–40-kD PrB is present. However, Fig. 9 D, which represents the CPY found in the very same extracts, suggests that the tunicamycin treatment was effective. Furthermore, the relative molecular mass of the sec18 PrB species (39 kD) is just about one-half the relative molecular mass of preproPrB (76 kD). Perhaps the amino-terminal polypeptide initially comigrates with the sec18 form of PrB, and is later degraded or secreted. A corollary of this hypothesis requires that the amino-terminal polypeptide is not glycosylated at its one Asn-linked glycosylation consensus sequence (Asn-Leu-Ser at -68). We have constructed a fusion between the anthranilate synthetase (TrpE) protein of *E. coli* and the amino-terminal portion of PrB. We have purified this fusion protein and are using it to affinity purify a subset of antibodies from the 77-kD ORF antiserum in order to address this problem more fully.

**Glycosylation of PrB and Its Precursors**

Glycosylation in yeast has recently been reviewed (33, 55, 58). Transfer from dolichol carriers of Glc₃Man₃GlcNAc₂ to asparagine residues, as well as Man, to serine and threonine residues, is known to occur in the ER (13, 18). The Asn-linked side chains are quickly trimmed to Man₃GlcNAc (in GLS1 strains), and then to the Man₃GlcNAc₂ form that accumulates in sec18-blocked cells (12). The hydroxy-linked side chains accumulate primarily as the Man₁₅ form during the sec18 block (18). Once secretory proteins reach the Golgi compartment, their carbohydrate moieties are elaborated. To the Asn-linked core are added 0–7 Man units for a total of 8–15 mannose residues per chain (60). Primarily for externally secreted proteins, the “core” is further elaborated in the Golgi compartment by adding outer chain segments of Man₁₄ up to totals as high as 150 residues per side chain (60). To the hydroxy-linked Man units, 0–3 mannose residues are added in the Golgi compartment (18).

PreproPrB and proPrB have both Asn-linked and non-Asn–linked carbohydrate; mature PrB only has non-Asn–linked carbohydrate. Fig. 9 B shows that one or more Asn-linked side chains are added to the 76-kD preproPrB in the ER. One Asn-linked chain is retained on all the processing intermediates, but no Asn-linked carbohydrate is present on the 31-kD mature form (Figs. 1, 2, 4, and 9). Figs. 3 and 4 demonstrate that only one or two (probably two) mannose units are added to the Man₃GlcNAc₂ (sec18) chain. Therefore, the Asn-linked moiety on prePrB in pep4 mutants exists as Man₃GlcNAc₂. Figs. 3 and 4 also demonstrate that either zero or one (probably zero) mannose units are added to the sum of the non-Asn–linked carbohydrate present on the 39-kD intermediate. Therefore, any non-Asn–linked (presumably hydroxy-linked) side chains on prePrB or mature PrB must exist as Man₁ units. This also is consistent with the lack of effect of the mnn1 mutation on prePrB or mature PrB. Mature PrB is assumed to have hydroxy-linked carbohydrate by the following argument. Previously, we reported a PrB purification scheme that used affinity chromatography over a Con A–Sepharose column and elution with α-methylmannoside (47). This approach was based on two earlier reports claiming that mature PrB contained 9% (32) and “no greater than 0.5%” carbohydrate (61). Mature protease B clearly has some form of mannose-containing carbohydrate, and since it cannot be Asn linked (this work; 43), we assume that it is hydroxy linked.

**Relative Half-Times for Maturation of Protease B and Carboxypeptidase Y**

Much work has been devoted to studying CPY as a model vacuolar protein. The transit and processing times for CPY have been well characterized (reviewed in reference 28). The transit time for CPY at 25°C was similar to that for PrB (Fig. 9). Fig. 9 also provides a clear depiction that CPY is probably cotranslationally modified and PrB is definitely posttranslationally modified.

**Studies of Vacuolar Targeting of Protease B**

As an outgrowth of investigations into the secretory process in yeast, there has been a great deal of interest in identifying both the gene products involved in protein sorting and the signals used by the cell for this sorting. Two groups have reported having mutants that are defective in the sorting of yeast vacuolar proteins (5, 53). Because it is known that yeast cells do not use mannose-6-phosphate for a vacuolar sorting signal the way mammalian cells do for lysosomal proteins (57), it is likely that the sorting signal is encoded in the primary or higher order sequence of the targeted protein. For that matter yeast do not even make the same type of mannose-6-phosphate units that mammalian cells do: yeast has an exposed R-Man-P-Man whereas animal cells have an exposed R-Man-P [19; reviewed in reference 63].) Through the use of a series of *prcl* mutants (62), and CPY-invertase (25) and PrA-invertase (31) protein fusions, defined segments of the propeptide sequences of proCPY and proPrA have been identified that are both necessary and sufficient for localization of these proteins to the vacuole.

The organization of the *PRB* ORF does not resemble that of the *PEP4* and *PRC1* ORFs, and the signals for PrB sorting do not appear to reside in homologous positions either. Recall that the processing of the 76-kD preproPrB to the 39-kD form occurs very rapidly in the ER, and that the first 280 amino acids of preproPrB are cleaved before it leaves the ER. The obvious conclusion to be drawn from this is that either the first 280 amino acids of preproPrB do not contribute to the sorting signal, or, the sorting decision for protease B occurs very early in the ER. A third possibility is that, although
the cleavage after residue 280 occurs in the ER, the two product polypeptides remain attached, possibly through a disulfide bond, and that PrB is targeted by its piggyback prepro region. There is no evidence in favor of the sorting decision occurring in the ER. With the possible exception of PEP7 (Garlow, S., and E. Jones, Carnegie Mellon University, unpublished data), there are no genes known that affect the secretion or processing of vacuolar proteins before entry into the vacuole, and yet fail to affect externally secreted proteins. In other words, both sets of secretory proteins seem to share the same processing machinery from ER translocation through to the late Golgi compartment (57).

The structure of PrB and its precursors raise several questions about protein processing and secretion in yeast; further studies of this protein should provide fresh insight into these processes. PrB may become a model protein for studies of translational—translational interactions in yeast. The large shift in the relative molecular mass of PrB that occurs in the ER, as opposed to the relatively small shift observed for other secretory proteins, makes this protein a useful probe in studies of ER function. Because proPrB has only one Asn-linked carbohydrate side chain, it is a better substrate for studying subtle aspects of this type of glycosylation than proteins having multiple side chains. Because mature PrB is relatively small and has only non-Asn-linked carbohydrate, and also because its structural gene has been cloned and sequenced, it should be a useful substrate for studies of non-Asn-linked glycosylation. Finally, further studies on the processing of PrB precursors may identify two new yeast processing proteases, one in the ER and one in the vacuole.

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