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Biogenesis of the yeast lysosome (vacuole): biosynthesis and maturation of proteinase yscB

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The biosynthesis and processing of the vacuolar (lysosomal) proteinase yscB was followed *in vivo* and *in vitro*. *In vitro* transcription-translation of the cloned proteinase yscB gene results in a high mol. wt precursor protein (HM_r-precursor) of M_r = 73 000. *In vivo* a precursor of identical mol. wt is found in *sec61* mutant cells deficient in translocation of secretory protein precursors into the lumen of the endoplasmic reticulum (ER). In contrast to *N*-glycosylated intermediate mol. wt forms of proteinase yscB, the HM_r-precursor of the enzyme is not *N*-glycosylated, indicating its appearance outside the ER. *sec18* Mutant cells, wild type for translocation of proteins into the ER but blocked in the delivery step of secretory proteins to the Golgi apparatus, accumulate a lower mol. wt precursor (LM_r-precursor) of proteinase yscB of M_r = 41 500. Processing of the HM_r-precursor of proteinase yscB to the LM_r-precursor requires proteolytic cleavage which is independent of the proteinase yscA gene product, a protein known to be involved in the processing event of the LM_r-precursor of proteinase yscB to the mature enzyme of M_r = 33 000. A LM_r-precursor of proteinase yscB of M_r = 42 000 accumulates in proteinase yscA-deficient mutant cells (allele *pep4-3*). This form is enzymatically active. Incubation *in vitro* of the LM_r-precursor of proteinase yscB with mature proteinase yscA leads to further activation and to processing of the enzyme yielding the mature 33 000 M_r protein. Processing is inhibited by the peptidase inhibitors pepstatin and chymostatin as well as by the specific proteinase yscB-inhibitor I_2^B, indicating that proteinase yscA as well as the LM_r-precursor of proteinase yscB itself are involved in the maturation process.

Key words: maturation/precursor/proteinase yscB/vacuoles/yeast

Introduction

The vacuole of the yeast *Saccharomyces cerevisiae* contains a multitude of hydrolases and has thus been assumed to be the lysosome of the yeast cell (Matile and Wiemken, 1976; Wiemken *et al.*, 1979). This view is strongly supported by the fact that vacuolar endopeptidases are highly unspecific and responsible for a large portion of intracellular protein degradation (Wolf, 1982; Jones, 1984; Achstetter and Wolf, 1985; Suarez Rendueles and Wolf, 1988). Seven peptidases have been shown to be vacuolar: the two endopeptidases proteinases yscA and yscB, the two carboxypeptidases yscY and yscS, the two aminopeptidases yscI and yscCo, and dipeptidyl aminopeptidase yscV (Achstetter and Wolf, 1985; Suarez Rendueles and Wolf, 1988). The import of carboxypeptidase yscY into the vacuole has been studied intensively (Stevens *et al.*, 1982; Johnson *et al.*, 1987; Valls *et al.*, 1987). As in mammalian cells (Erickson *et al.*, 1984) the import pathway leads from the site of synthesis, the endoplasmic reticulum, through the Golgi apparatus to the vacuole. All soluble vacuolar peptidases analyzed in more detail are synthesized as higher mol. wt precursors (Hasilik and Tanner, 1978; Mechler *et al.*, 1982a) which were reported to be inactive (Hasilik and Tanner, 1978; Hemmings *et al.*, 1981; Jones *et al.*, 1982). Pulse-chase experiments identified precursor molecules of M_r = 52 000 for proteinase yscA, M_r = 42 000 for proteinase yscB and M_r = 67 000 for carboxypeptidase yscY yielding the mature proteins of M_r = 42 000, M_r = 33 000 and M_r = 61 000 respectively (Mechler *et al.*, 1982a; Hasilik and Tanner, 1978). Maturation of carboxypeptidase yscY has been studied in some detail. Using conditional secretion deficient mutants (*sec*) it could be shown that processing of the enzyme occurs in transit from the Golgi apparatus to the vacuole, or in the vacuole itself (Stevens *et al.*, 1982). Processing occurs via peptidolytic cleavage of an amino-terminal peptide (Hemmings *et al.*, 1981; Valls *et al.*, 1987). *In vivo* and *in vitro* experiments identified mature proteinase yscA (Ammerer *et al.*, 1986; Woolford *et al.*, 1986; Mechler *et al.*, 1987) and mature proteinase yscB (Mechler *et al.*, 1987) as the processing and activating catalysts. A mutation in the proteinase yscA gene (allele *pep4-3*) not only leads to accumulation of the carboxypeptidase yscY precursor protein (Hemmings *et al.*, 1981) but also to the accumulation of the 42 000 M_r precursor of proteinase yscB, indicating involvement of the proteinase yscA gene product in maturation of this protein (Mechler *et al.*, 1982b). Recent cloning and sequencing of the proteinase yscB structural gene revealed an ORF of 635 codons which might encode a protein of >69 000 M_r (Moehle *et al.*, 1987a,b). We have also cloned the proteinase yscB gene and we show here that its transcription and translation *in vitro* yields a protein of M_r = 73 000. We follow biosynthesis of proteinase yscB *in vivo*, its transport through part of the secretory pathway, and we report studies on the processing and activation mechanism of the enzyme.

Results

In vitro transcription-translation of the gene encoding proteinase yscB

The proteinase yscB gene was cloned by complementation of a mutation in the structural gene of the enzyme (*prb1-1*) isolated some time ago (Wolf and Ehmann, 1978,1979,1981;

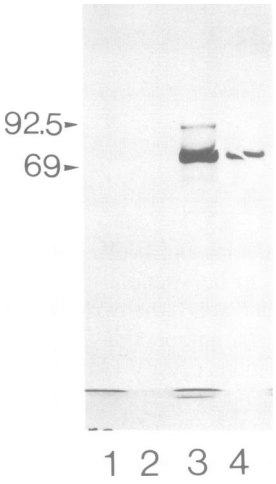

Fig. 1. Proteinase yscB is synthesized as a high mol. wt precursor *in vitro*. *In vitro* transcription and translation of pSP64/PRB1 and immunoprecipitation was done as outlined in Materials and methods. SDS−PAGE (10% gel) and fluorography were done as outlined by Mechler *et al.* (1982a). **Lane 1**, 12.5 μl translation mixture without transcription mixture added (control); **lane 2**, 12.5 μl translation mixture without transcription mixture added, immunoprecipitated with proteinase yscB antiserum (5 μl) (control); **lane 3**, 12.5 μl translation mixture containing 0.5 μl transcription mixture; **lane 4**, 12.5 μl translation mixture containing 0.5 μl transcription mixture, immunoprecipitated with proteinase yscB antiserum (5 μl).

Mechler *et al.*, 1982b). Cloning was done with a genomic *S.cerevisiae* DNA library inserted into the centromere shuttle vector pCS19 (Sengstag and Hinnen, 1987). Restriction maps of the three complementing plasmids isolated, yielded identical DNA fragments able to restore proteinase yscB activity as described by Moehle *et al.* (1987a). Sequencing of part of a 2.3-kb *Pst*I−*Xho*I DNA fragment complementing proteinase yscB activity in mutants showed that the isolated gene was indeed identical with the gene isolated and sequenced by Moehle *et al.* (1987b) (not shown). The ORF of *PRB1* consists of 635 codons and thus possibly encodes a protein of >69 000 M_r (Moehle *et al.*, 1987b) as compared to the mature form of the enzyme of M_r = 33 000 (Kominami *et al.*, 1981; Mechler *et al.*, 1982a). Pulse-chase experiments had identified a precursor of proteinase yscB of M_r = 42 000 in yeast cells, which is transferred into its 33 000 M_r mature form (Mechler *et al.*, 1982a). No indication had been found in wild-type yeast cells of the existence of a >69 000 HM_r-precursor form of the enzyme. To determine whether a high mol. wt precursor form of proteinase yscB is synthesized *in vitro* upon transcription of the isolated *PRB1* gene and translation of the resulting mRNA, we inserted the *Afl*II−*Xho*I fragment into the transcription vector pSP64 under control of the SP6 RNA polymerase promotor. Transcription of the linearized plasmid DNA, translation of the resulting mRNA in a reticulocyte lysate in the presence of [^{35}S]methionine and separation of the translation products on SDS−PAGE showed a prominent protein of M_r = 73 000 (Figure 1, lane 3). This product can be precipitated with antibodies against mature proteinase yscB, identifying it as a proteinase yscB precursor protein (Figure 1, lane 4).

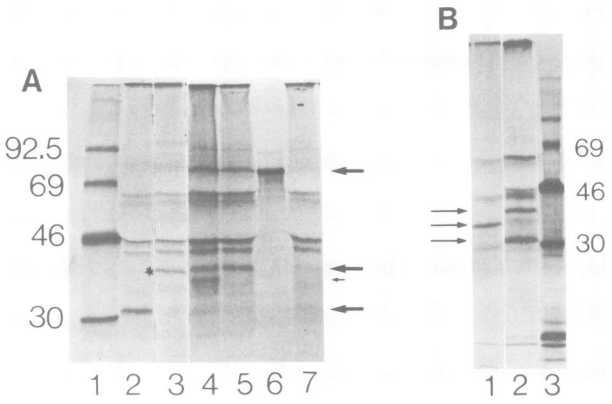

Fig. 2. Occurrence of a high and a lower mol. wt precursor of proteinase yscB in the secretory pathway. Growth of cells, labeling of proteins, immunoprecipitation of proteinase yscB immunoreactive material with proteinase yscB antiserum, SDS−PAGE and fluorography were done as outlined in Materials and methods. (**A**) (10% gel) Upper large arrow indicates position of the HM_r-precursor of proteinase yscB; intermediate large arrow indicates position of the LM_r-precursor of proteinase yscB accumulating in strains defective in proteinase yscA (allele *pep4-3*). Lower large arrow indicates position of mature proteinase yscB. Small arrow indicates position of the LM_r-precursor of proteinase yscB accumulating in tunicamycin-treated strains carrying the proteinase yscA deficiency (allele *pep4-3*). Asterisk indicates position of the LM_r-precursor accumulating in strain carrying the *sec18* mutation. **Lane 1**, marker proteins; **lane 2**, wild-type strain S288C; **lane 3**, strain SECY18-1D (*sec18*); **lane 4**, strain RDM15-5B (*sec61 pep4-3*) in the presence of tunicamycin (74 μg/ml); **lane 5**, strain RDM15-5B (*sec61 pep4-3*); **lane 6**, *in vitro* translated proteinase yscB HM_r-precursor as in lane 3 of Figure 1; **lane 7**, experiment as in lane 5 but with the addition of 245 μg proteinase yscB prior to immunoprecipitation to compete for radiolabeled proteinase yscB antigenic material. (**B**) (12% gel) Upper arrow indicates position of LM_r-precursor of proteinase yscB. Intermediate arrow indicates position of LM_r-precursor of proteinase yscB accumulating in the strains carrying the *sec53* mutation. Lower arrow indicates position of mature proteinase yscB. **Lane 1**, strain MM8 (*sec53*); **lane 2**, strain MM1, wild type; **lane 3**, marker proteins.

In vivo biosynthesis of proteinase yscB in different secretion deficient (sec) mutants

As such an HM_r-precursor had never been found in wild-type cells, the question of its *in vivo* biosynthesis, its translocation pathway into the vacuole and the question of the maturation events have to be answered. The knowledge that the vacuolar carboxypeptidase yscY traverses part of the secretory pathway to reach the vacuole (Stevens *et al.*, 1982) made it highly probable that transport of proteinase yscB follows the same pathway. Elucidation of the traffic of secreted proteins in yeast is facilitated by using mutant strains conditionally defective in crucial steps of the secretory pathway (Schekman, 1982,1985). The recently isolated *sec61* mutation, which makes strains conditionally defective in an early cytoplasmic or ER membrane-associated step in protein translocation (Deshaies and Schekman, 1987), the *sec18* mutation which conditionally blocks the delivery of secreted proteins from the ER to the Golgi apparatus (Novick *et al.*, 1980,1981), and the *sec53* mutation, leading to conditional accumulation of defectively glycosylated secretory proteins in the ER of strains (Feldman *et al.*, 1987), allowed dissection of steps in proteinase yscB biosynthesis, transport and maturation.

The *sec61* mutant cells used (strain RDM 15-5B) in addition carried a mutation in proteinase yscA (allele *pep4-3*) preventing processing of the M_r = 42 000 precursor protein of proteinase yscB to the mature form (Mechler *et al.*, 1982b). *sec61* cells exhibit their mutant phenotype, which is not stringently expressed, at 30 and 37°C (Deshaies and Schekman, 1987). When labeled with [^{35}S]methionine at these temperatures, two precursors of proteinase yscB can be detected after immunoprecipitation and SDS−PAGE (Figure 2A, lane 5, upper two large arrows; for comparison, mature proteinase yscB of wild-type cells is seen in Figure 2A, lane 2, lower large arrow). One precursor of M_r = 73 000 and one of M_r = 42 000 is precipitated. The additional radioactive proteins of intermediate mol. wt visible on the gel are not due to proteinase yscB antigens and must be considered as contaminants as only the two molecules of M_r = 73 000 and M_r = 42 000 compete with mature proteinase yscB for the proteinase yscB antibody (Figure 2A, lane 7). The HM_r-precursor of M_r = 73 000 precipitated from the mutant cells is of identical size to the *in vitro* translated proteinase yscB precursor (Figure 2A, lane 6). As expected from the proteinase yscA mutation (allele *pep4-3*) carried by the strain used, the LM_r-precursor, which must have escaped the *sec61* block, has a mol. wt of 42 000.

A proteinase yscB precursor, which has been translocated into the ER or has passed this organelle, is expected to carry at least one *N*-linked carbohydrate chain (Mechler *et al.*, 1982a). Tunicamycin, a specific inhibitor of *N*-glycosylation (Elbein, 1981) does not affect the mol. wt of the HM_r-precursor of proteinase yscB (Figure 2A, lane 4), indicating that this molecule has not entered the ER. This is in perfect agreement with the general behaviour of secreted proteins in *sec61* mutant cells: they do not pass the ER membrane and thus are not glycosylated (Deshaies and Schekman, 1987). In contrast, the LM_r-precursor of proteinase yscB of M_r = 42 000 is affected by tunicamycin treatment, resulting in a 39 000 M_r molecule (Figure 2A, lane 4, small arrow); therefore the only *N*-linked carbohydrate chain of this molecule (Mechler *et al.*, 1982a) is missing.

When biosynthesis of proteinase yscB was studied in *sec18* mutant cells at non-permissive temperature, allowing entry of secretory proteins into the ER but blocking their transfer into the Golgi apparatus (Novick *et al.*, 1980,1981), the LM_r-precursor of proteinase yscB accumulates (Figure 2A, lane 3, asterisk). No HM_r-precursor was detectable. The LM_r-precursor was found to have a slightly reduced mol. wt (M_r = 41 500) as compared to the precursor in processing deficient proteinase yscA (allele *pep4-3*) mutant cells. This behaviour may indicate a glycoprotein which has not yet undergone trimming on passage through the Golgi apparatus (Stevens *et al.*, 1982), in contrast to the 42 000 M_r precursor accumulating in the vacuole of proteinase yscA (allele *pep4-3*) mutant cells (Figure 2A, lanes 4 and 5; Figure 4, lane 1). Also in *sec53* mutant cells, in which secretory proteins accumulate in the ER due to interference with glycosylation (Feldman *et al.*, 1987), only the LM_r-precursor of proteinase yscB accumulates under restrictive conditions (Figure 2B, lane 1, intermediate arrow). As expected, the mol. wt of this precursor is identical (within the limits of the method employed) to the one emerging in cells carrying the proteinase yscA mutant allele *pep4-3* and treated with tunicamycin (Figure 2A, lane 4). This indicates that interference with glycosylation does not prevent processing of the HM_r-precursor.

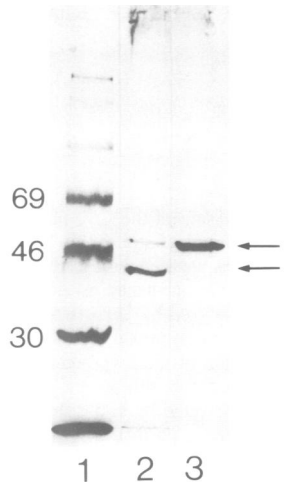

Fig. 3. Proteinase yscA also accumulates as a precursor in the ER. Growth of cells, labeling of proteins, immunoprecipitation of proteinase yscA immunoreactive material, SDS−PAGE (12% gel) and fluorography were done as outlined in Materials and methods. Upper arrow indicates position of proteinase yscA precursor; lower arrow indicates position of mature proteinase yscA. **Lane 1**, marker proteins; **lane 2**, strain X2180-1A, wild type; **lane 3**, strain SECY18-1D (sec18).

Taken together, these studies show that proteinase yscB is synthesized as an HM_r-precursor of M_r = 73 000 which, upon entry into the ER, undergoes proteolytic processing yielding the LM_r-precursor detected previously (Mechler *et al.*, 1982a).

Further proteolytic cleavage of the LM_r-precursor yields mature proteinase yscB of M_r = 33 000 (Mechler *et al.*, 1982a). This final maturation step is defective in *pep4-3* mutant cells (Mechler *et al.*, 1982b) and thus dependent on a product of the proteinase yscA gene (Ammerer *et al.*, 1986; Woolford *et al.*, 1986). We further investigated this proteinase yscA-dependent processing step. As Figure 3 shows, in *sec18* mutant cells, proteinase yscA also accumulates as a precursor molecule under restrictive conditions. This indicates that proteinase yscA travels together with the proteinase yscB along part of the secretory pathway. As no maturation of the LM_r-precursor of proteinase yscB occurs in *sec18* mutant cells, we consider the precursor of proteinase yscA not to be the activating molecule under the conditions present in the ER.

In vitro activation and processing of the LM_r-precursor of proteinase yscB

In vivo studies had shown that a mutation in the structural gene of proteinase yscA (allele *pep4-3*) prevented processing of the LM_r-precursor of proteinase yscB, indicating the involvement of proteinase yscA in this process. We set up an *in vitro* system to shed some light into this activation event. Vacuoles of the proteinase yscA mutant strain carrying the *pep4-3* allele were isolated and used as source of the LM_r-precursor of proteinase yscB (Mechler *et al.*, 1982b).

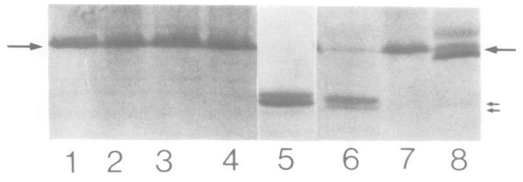

Fig. 4. Activation and processing of the LM_r-precursor of proteinase yscB *in vitro*. Vacuoles (140 μg) of strain 20B-12 (pep4-3) deficient in proteinase yscA were used as source of the LM_r-precursor of proteinase yscB. Vacuoles were incubated as indicated below. Incubation mixtures were analyzed by SDS–PAGE (10% gel) followed by immunoblotting as described in Materials and methods. Upper arrows indicate position of the LM_r-precursor of proteinase yscB. The two lower small arrows indicate positions of two mature proteinase yscB proteins as isolated by two different isolation procedures. [Upper small arrow, purification after Kominami *et al.* (1981); lower small arrow, purification after Betz *et al.* (1974)]. **Lane 1**, vacuoles in 0.1 M Tris–maleate buffer, pH 5; **lane 2**, vacuoles as in lane 1, but after 5 h incubation at 30°C; **lane 3**; vacuoles in 0.1 M Tris–maleate buffer, pH 7.5; **lane 4**, vacuoles as in lane 3, but after 5 h incubation at 30°C; **lane 5**, the two mature proteinase yscB preparations purified after Kominami *et al.* (1981) (7 μg) and Betz *et al.* (1974) (24 μg); **lane 6**, vacuoles incubated for 5 h at 30°C in 0.1 M Tris–maleate buffer, pH 5, in the presence of purified proteinase yscA (6 μg); **lane 7**, vacuoles, incubated as in lane 6, but in the presence of pepstatin (1 μg); **lane 8**, vacuoles, incubated as in lane 6, but in the presence of chymostatin (2 μg).

Unexpectedly, this precursor molecule shows a considerable specific activity of ~0.15 against the proteinase yscB substrate Azocoll. This activity increases ~2-fold (specific activity: 0.3) upon incubation (5 h, 30°C) without any further additions at pH 5 or pH 7.5. However, no processing to the mature 33 000 M_r proteinase yscB occurs during this activation process (Figure 4, compare lanes 1 and 2 as well as 3 and 4 with lane 5; the double band in lane 5 represents a mixture of two independently purified proteinase yscB preparations). The activity appearing during this incubation can be inhibited by the proteinase yscB inhibitor chymostatin or the specific proteinase yscB inhibitor I_2^B (not shown). Thus, the lower mol. wt proteinase yscB precursor is active: the activity is, however, masked. Whether the release of this cryptic activity during incubation is due to dissociation or digestion of some inhibitory molecule or due to some other event is still unknown. Processing of the LM_r-precursor of proteinase yscB to the mature protein is achieved by addition of purified yscA (Figure 4, lane 6). This processing step leads to an additional activation of proteinase yscB of ~2-fold over the 'activated' LM_r-precursor (specific activity: 0.7). Optimum pH for processing is pH 5. No processing is observed above pH 6.5 (not shown). The *in vitro* processing event leads to two mature proteinase yscB proteins similar to the proteinase yscB proteins that can be obtained by purification (Figure 4, lanes 5 and 6). As expected, if one assumes catalytic action of proteinase yscA in the processing event, the maturation of the lower mol. wt proteinase yscB precursor is inhibited by pepstatin, a specific inhibitor of proteinase yscA (Figure 4, lane 7). Surprisingly, however, maturation of the precursor is also inhibited by chymostatin, an inhibitor of proteinase yscB (Figure 4, lane 8) as well as by the specific proteinase yscB inhibitor I_2^B (not shown). Only a small shift in mol. wt of the precursor is detectable under these conditions (Figure 4, lane 8). This indicates that both proteins, proteinase yscA and the LM_r-precursor of proteinase yscB itself, are involved in the processing event.

Discussion

Using secretion-deficient mutants (Schekman, 1982,1985; Deshaies and Schekman, 1987), we showed that proteinase yscB traverses part of the secretory pathway to reach its final destination, the vacuole. The enzyme can be synthesized as an HM_r-precursor protein of M_r = 73 000 *in vitro*. By kinetic pulse–chase studies such a molecule could not be found in wild-type yeast cells (Mechler *et al.*, 1982a), nor was it detectable in mutants (*sec18*) conditionally defective in transport of secretory proteins from the ER to the Golgi apparatus by accumulating them in the lumen of the ER (Figure 2A, lane 3). Only the LM_r-precursor of proteinase yscB found previously (Mechler *et al.*, 1982a) is detectable under these conditions. The 73 000 M_r precursor was found, however, *in vivo*, but only in mutants (*sec61*) conditionally defective in the translocation of secretory proteins into the ER (Figure 2A, lane 5). This indicates that the HM_r-precursor of proteinase yscB undergoes proteolytic cleavage upon translocation into the ER or in the lumen of the ER to yield the LM_r-precursor. The processing proteinase is at present unknown.

The LM_r-precursor of proteinase yscB contains one *N*-linked carbohydrate chain, which is removed during the proteolytic maturation process (Mechler *et al.*, 1982a). The somewhat slower electrophoretic mobility of the LM_r-precursor accumulating in vacuoles of processing-deficient proteinase yscA mutant cells (Mechler *et al.*, 1982b; Figure 2A, lanes 4 and 5), as compared to the ER form (Figure 2A, lane 3), is most likely due to carbohydrate modification occurring in the Golgi apparatus.

Rather unexpectedly, the LM_r-precursor of proteinase yscB is proteolytically active against Azocoll. The activity of this precursor can be significantly increased by incubating vacuoles from the processing-deficient proteinase yscA mutant (allele *pep4-3*) for several hours at pH 5 or pH 7.5. During this treatment proteolytic activity reaches about one-half of that of mature proteinase yscB without any detectable alteration of the mol. wt of the precursor molecule (Figure 4, lanes 1–4). We do not know the mechanism of this activation event. The activation mechanism might reside in the removal of some small peptide of the precursor by self-cleavage, which does not alter the mol. wt of the precursor significantly and thus remains undetectable by SDS–PAGE. Alternatively, activation might be brought about by dissociation or digestion of a non-covalently bound inhibitor protein. No function has been reported for the peptide sequence cleaved off the HM_r-precursor of proteinase yscB. It is attractive to speculate that this peptide functions as the inhibitor of the LM_r-precursor in the secretory pathway to prevent damage which might otherwise be caused by the unspecific degradative activity of this precursor.

Proteinase yscA is involved in proteolytic processing of the LM_r-precursor of proteinase yscB to yield the mature 33 000 M_r enzyme (Mechler *et al.*, 1982b; Ammerer *et al.*, 1986; Woolford *et al.*, 1986). Proteinase yscA travels together with proteinase yscB through compartments of the secretory pathway. The proteinase yscA precursor does not activate the LM_r-precursor of proteinase yscB under the conditions present in the ER (Figure 2A, lane 3). As can be shown by *in vitro* experiments, mature proteinase yscA leads to processing of the LM_r-precursor of proteinase yscB under moderately acidic conditions (Figure 4, lane 6). The optimum pH (pH 5) is identical to the optimum pH for

maturation of another vacuolar enzyme, carboxypeptidase yscY (Mechler *et al.*, 1987). Inhibition of the processing event of the LM_r-precursor by pepstatin (Figure 4, lane 7), a specific inhibitor of proteinase yscA, might imply that proteinase yscA is the only protein catalytically involved in processing. However, chymostatin and I_2^B, specific inhibitors of the activity of mature proteinase yscB as well as of the activity of the LM_r-precursor of the enzyme, also prevented processing of the LM_r-precursor to the authentic, mature enzyme. Only a small reduction in size of the precursor could be observed (Figure 4, lane 8). This indicates that in addition to proteinase yscA, the LM_r-precursor of proteinase yscB itself is involved in its own maturation process. One might consider the following different interpretations to explain the facts observed.

(i) Proteinase yscA cleaves in a purely catalytic step the LM_r-precursor of proteinase yscB yielding a protein of somewhat higher mobility on SDS−PAGE. This proteolytic step is inhibited by pepstatin (Figure 4, lanes 8 and 7). The intermediate form generated is further processed by self-maturation. This step is inhibited by chymostatin and I_2^B.

(ii) This model does not, however, account for the finding that proteinase yscB activity shows gene dosage which is strongly dependent on the copy number of the proteinase yscA gene (Jones *et al.*, 1982; Woolford *et al.*, 1986). One explanation for this phenomenon might be formation of a stoichiometric complex between proteinase yscA and the LM_r-precursor of proteinase yscB. Processing of the proteinase yscB precursor should occur only in this complex. Processing in the vacuole would require a permanently stable complex of proteinase yscA with proteinase yscB even after processing has occured. Complex formation between proteinase yscA and proteinase yscB has been shown by Hinze *et al.* (1975). If processing of the precursor was restricted to the transit of the protein from the Golgi apparatus to the vacuole, stoichiometric complex formation between proteinase yscA and the LM_r-precursor of proteinase yscB would only be required during the transit step. Processing in the complex could occur via the catalytic action of proteinase yscA and the LM_r-precursor of proteinase yscB. The inhibitors peptstatin, chymostatin and I_2^B might block these activities in the complex. Blockage of the proteinase yscB maturation might also be due to interference of one or the other of the inhibitors with complex formation.

Materials and methods

Strains

Escherichia coli strains HB 101(*F⁻, hsdS20, recA13 ara14, proA2, lacY1, galK2, straA xyl-5, mtl-1, SupE44*) and JM109 (*recA1, endoA1, gyrA96, thi⁻, hsdR17, supE44, relA1 (lac,pro) F'traD36 proAB lacI^q, lacZ ΔM15*) (Miller, 1987) were used as hosts for plasmids. Wild-type yeast strains were S288C (α) and X2180-1A (*a*) (Yeast Genetic Stock Center, Berkeley, CA) and MM1 (B.Mechler and H.Müller, unpublished). Proteinase yscA-deficient mutant strain was strain 20B-12 (α *pep4-3 trp1*) (Hemmings *et al.*, 1981; Yeast Genetic Stock Center, Berkeley, CA). Conditional secretory deficient strains were strain RDM15-5B (α *sec61-2 pep4-3 leu2-3, 112 ade2 ura3-52*) (Deshaies and Schekman, 1987), SECY18-1D (*a sec18-1 prc1*) (Novick *et al.*, 1981; Emter *et al.*, 1983) and MM8 (*sec53*) (Feldman *et al.*, 1987) originating from a cross of strain RH234 (α *sec53 his4*) and strain C12-ABYS91 (*a pra1-1 prb1-1 prc1-1 cps1-3 his lys leu2 trp1*) (B.Mechler and H.Müller, unpublished). Proteinase yscB-deficient mutant strain was strain HP232-5D (α *prb1-1 cly4 ura1 lys2 tyr1 ade⁻*) (Wolf and Ehmann, 1979). Cloning of the proteinase yscB gene by complementation was done using strain YHH2 (*a prb1-1 cly4 trp1 ura3Δ5*) a derivative of strain HP232-5D (H.Hirsch, unpublished).

Media

For growth of *E.coli* strains LB medium (Maniatis *et al.*, 1982) with or without ampicillin (100 mg/l) or tetracylcin (12.5 mg/l) was used as complete medium.

Yeast growth media were YPD-complete medium (1% yeast extract, 2% peptone, 2% glucose) and mineral medium (0.67% yeast nitrogen base without amino acids, 2% or 5% glucose, supplements required by auxotrophic strains). For labeling experiments mineral medium without ammonium sulfate (0.17% yeast nitrogen base without ammonium sulfate, supplements required by auxotrophic strains, 0.1% yeast extract, 1.5 or 0.3% glucose, pH 6) was used. Additional media were YPG (1% yeast extract, 2% peptone, 3% glycerol) and proteinase yscB-mutant selection medium, YHS (0.5% yeast extract, 1% peptone, 1% glucose, 2% Hide Powder Azure, 0.05% SDS, pH 7).

Enzymes and inhibitors

Restriction enzymes were obtained from Boehringer Mannheim or Stehelin AG, Basel. T4 DNA ligase was from Biolabs, Beverly, MA. The large Klenow DNA polymerase I fragment was obtained from Bethesda Research Laboratories, Basel. Vectors pGEM2 and pSP64, SP6 RNA polymerase and rabbit reticulocyte lysate were from Promega Biotec, Madison, WI. Proteinase yscA was purchased from Sigma, Deisenhofen. Chymostatin and pepstatin were from the Peptide Institute, Osaka. Proteinase yscB purified after Kominami *et al.* (1981) was a gift from H.Hoffschulte, Freiburg, proteinase yscB purified after Betz *et al.* (1974) was a gift from U.Weiser, Freiburg. Proteinase yscB inhibitor I_2^B was a gift of Dr P.Bünning, Freiburg.

Immune sera

Immune sera against proteinase yscA and proteinase yscB had been described and characterized previously (Mechler *et al.*, 1982a).

Enzyme assays

Proteinase yscA was tested against acid hemoglobin as substrate, proteinase yscB as well as the LM_r-precursor of proteinase yscB were tested using Azocoll as substrate. Tests were done as described (Saheki and Holzer, 1974). Specific activity of proteinase yscB is given as absorption at 520 nm/min × mg protein.

Molecular cloning

Procedures followed standard protocols (Maniatis *et al.*, 1982). Competent *E.coli* strains were obtained using the standard $CaCl_2$ method (Maniatis *et al.*, 1982). Yeast transformation was carried out as described by Ito *et al.* (1983).

Genomic library and plasmids

The genomic library of *S.cerevisiae* in the *ARS1 CEN15 URA3* vector pCS19 has been described (Sengstag and Hinnen, 1987). For subcloning of complementing fragments, the *ARS1 CEN14 URA3* vector pDP39 (Sengstag and Hinnen, 1987; R.D.Pridmore, unpublished results) was used. For single-strand sequencing, DNA fragments were cloned into the vector pEMBL18 (Dente *et al.*, 1983) and single-strand (ss) DNA was prepared after infection of transformed *E.coli* strain JM109 with the helper phage R408 (Russel *et al.*, 1986) (Stratagene cloning Systems, San Diego, CA). For *in vitro* transcription, the proteinase yscB gene was cloned into the vectors pGEM2 and pSP64.

Isolation of the proteinase yscB gene

The structural gene of proteinase yscB was cloned using a genomic library contained in the centromere shuttle vector pCS19 (Sengstag and Hinnen, 1987) by complementation of the previously characterized mutation *prb1-1* (Wolf and Ehmann, 1978,1979). Plasmid DNA of the library was purified according to standard procedures (Maniatis *et al.*, 1982) and used to transform the yeast strain YHH2 according to Ito *et al.* (1983). Uracil prototrophic transformants were selected at 25°C. Transformants were replica-plated on YPG plates and incubated for 72 h at 25°C. For identification of complementing transformants, the colonies were replica-plated onto proteinase yscB indicator plates YHS and incubated at 37°C to induce lysis (Wolf and Ehmann, 1978,1979). Clear zones in the blue Hide Powder Azure particles around transformants indicated release of proteinase yscB. Among 15 000 transformants three positive clones were identified. After transformation of *E.coli* strain HB101 with total yeast DNA of these clones, the plasmids were purified. Retransformation of the yeast strain YHH2 proved that the observed complementation was linked to the presence of the genomic yeast DNA insert. The restriction maps revealed that the genomic inserts of all three plasmids contained an identical complementing DNA subfragment. This fragment was identical to the one recently described as containing the structural gene of proteinase yscB (Moehle *et al.*, 1987a). The smallest fragment still capable of complementing the *prb1* mutation was found to be a 2.3-kb *Pst*I−*Xho*I fragment. This

fragment was cloned into the vector pEMBL18 for dideoxy sequencing (Sanger *et al.*, 1977). Sequencing data confirmed identity of the proteinase yscB gene isolated with the recently published *PRB1* sequence of Moehle *et al.* (1987b) (not shown).

Construction of the in vitro transcription vector pSP64/PRB1

The 2.3-kb *PstI*–*XhoI PRB1* fragment was ligated into the *PstI*- and *SalI*-digested vector pGEM2 (pGEM2/PXB). In order to eliminate potential start codons other than the correct ATG of the ORF of *PRB1*, the DNA sequence upstream of the *AflII* site was deleted by digesting pGEM2/PXB with *HindIII* and *AflII*, followed by end repair and blunt end ligation. Subsequently the *AflII*–*XhoI* fragment of *PRB1* was cloned as *HindIII*–*XbaI* fragment into the respective sites of the vector pSP64 to result in pSP64/PRB1. Dideoxy sequencing of the vector insert junction confirmed that the ORF of proteinase yscB was put correctly under the promoter control of the SP6 RNA polymerase.

In vitro transcription

pSP64/PRB1 was linearized downstream of the ORF by digestion with *XbaI*. Linearized DNA (1.0 μg) was transcribed *in vitro* at 40°C for 60 min using 10 U of SP6 RNA polymerase in a final reaction volume of 25 μl as described by the suppliers (Promega Biotec), resulting in uncapped RNA. The reaction mixture was stored frozen at −80°C.

In vitro translation

Cell-free translation was carried out at 37°C for 60 min using 10 μl of 'treated' rabbit reticulocyte lysate (Promega Biotec), 10 μCi of [^{35}S]methionine in a final volume of 25 μl with or without addition of 1.0 μl of the transcription mixture. Aliquots of 12.5 μl of each sample were taken and directly solubilized in SDS sample buffer for 4 min at 95°C. The residual of both samples was used for immunoprecipitation. Immunoprecipitation of *in vitro* translated proteins followed the procedure of Anderson and Blobel (1983) with the following modifications. After addition of SDS yielding a final concentration of 4% and heating to 100°C for 4 min, the samples were diluted with 1.0 ml of TNET buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 5 mM EDTA). 10.0 μl of 100 mM PMSF, 5.0 μl of proteinase yscB antiserum and 10 mg of protein A-sepharose 4B were added and shaken overnight at 4°C. After washing the precipitate three times with TNET and once with NET (buffer as above, but without Triton X-100), 30 μl of SDS sample buffer (100 mM Tris–HCl, 4% mercaptoethanol, 10% glycerol) were added for solublization. The mixture was treated 4 min at 100°C. After a 30-s centrifugation, the supernatants were transferred into new tubes. All samples were analyzed by SDS–PAGE (10% gel) and fluorography.

In vivo biosynthesis of proteinase yscB

Labeling of cell proteins was performed with [^{35}S]methionine (300 μCi/ml). Cells were shifted from glucose (5%) mineral medium to low glucose containing mineral medium without ammonium sulfate containing [^{35}S]methionine. Wild-type S288C and strain RDM15-5B were labeled for 1 h at 30°C and for an additional hour at 37°C. Strain SECY18-1D was labeled for 2 h at 37°C. Wild-type strain MM1 and mutant strain MM8 were labeled for 3 h at 37°C. Incubation with tunicamycin was done for 10 min prior to labeling of cells. Preparation of extracts, immunoprecipitation of proteins, SDS–PAGE and fluorography were done as outlined by Mechler *et al.* (1982a). ^{14}C-Methylated marker proteins used were: phosphorylase b (92.5 kd), bovine serum albumin (69 kd), ovalbumin (46 kd) and carbonic anhydrase (30 kd).

Isolation of vacuoles

Spheroplast formation and isolation of vacuoles was done as described (Wiemken *et al.*, 1979) with modifications as outlined by Mechler *et al.* (1987). Enrichment of vacuoles was ~10-fold.

Processing of proteinase yscB in vitro

Vacuoles of strain 20B-12 containing pro-proteinase yscB were incubated as described in the legend to Figure 4. The mixtures were tested for activity as well as subjected to TCA prepcipitation (5%) followed by SDS–PAGE. Gels were subjected to electroblotting onto nitrocellulose filters with subsequent decoration of the filters with specific antibodies. Visualization of immunoreactive protein was done as outlined by Mechler *et al.* (1987).

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