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Maturation of vacuolar (lysosomal) enzymes in yeast: proteinase yscA and proteinase yscB are catalysts of the processing and activation event of carboxypeptidase yscY

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Studies were performed to unravel the activation and maturation mechanism of vacuolar (lysosomal) proteinases in *Saccharomyces cerevisiae*. *In vivo* and *in vitro* studies show that proteinase yscA and proteinase yscB are involved in the activation and processing event of pro-carboxypeptidase yscY. Processing and activation of pro-carboxypeptidase yscY by proteinase yscA depends on an additional factor contained in the vacuolar fraction. Comparable activation can be mimicked by sodium polyphosphate. Optimum pH for processing by this proteinase yscA-triggered event is 5. The proteinase yscA-triggered maturation process of pro-carboxypeptidase yscY leads to an intermediate mol. wt form of the enzyme which is, however, fully active. Proteinase yscB transfers the intermediate mol. wt form of the original precursor to the apparently authentic, mature and active carboxypeptidase yscY. An activation and maturation scheme is devised.

Key words: maturation/vacuole/lysosome/pro-enzymes/yeast

Introduction

The vacuole of the yeast *Saccharomyces cerevisiae* has been shown to be the organelle containing a multitude of hydrolytic enzymes and consequently has been proposed to be the lysosome of the yeast cell (Matile and Wiemken, 1967; Wiemken *et al.*, 1979). The biosynthesis and function of a variety of vacuolar proteinases has been studied (Wolf, 1982, 1986; Jones, 1984; Achstetter and Wolf, 1985a). Three vacuolar enzymes, proteinase yscA (M_r = 42 000), proteinase yscB (M_r = 33 000) and carboxypeptidase yscY (M_r = 61 000), involved in protein degradation and essential for sporulation, have been shown to be synthesized as higher mol. wt precursors of M_r = 52 000 (proteinase yscA), 42 000 (proteinase yscB) and 67 000 (carboxypeptidase yscY), which undergo proteolytic processing to yield the mature enzymes (Hasilik and Tanner, 1978; Mechler *et al.*, 1982a).

In the case of carboxypeptidase yscY, biosynthesis and transport into the vacuole has been studied in some detail. As in mammalian cells (Erickson *et al.*, 1984), the transport pathway is defined as leading from the site of synthesis, the endoplasmic reticulum, through the Golgi apparatus to the lysosomal compartment, the vacuole. Proteolytic cleavage of the propeptide of the inactive pro-carboxypeptidase yscY molecule occurs either in transit of the Golgi body or after the proenzyme arrives at the vacuole (Stevens *et al.*, 1982).

In earlier experiments it had been shown that proteinase yscB was able to activate pro-carboxypeptidase yscY *in vitro* (Hasilik and Tanner, 1978). However, mutants devoid of proteinase yscB activity exhibited normal proteinase yscA and carboxypeptidase yscY activities (Wolf and Ehmann, 1978,1979; Zubenko *et al.*, 1979) which seemed to indicate that this enzyme is not involved in the processing event *in vivo*.

A mutant (*pep4-3*) had been isolated, which leads to the deficiency of a variety of vacuolar enzyme activities (Jones, 1977; Jones *et al.* 1981, 1982). *pep4-3* cells accumulate inactive precursor forms of proteinase yscB (Mechler *et al.*, 1982b) and carboxypeptidase yscY (Hemmings *et al.*, 1981). This finding has led to the assumption that the *PEP4* gene product is the proteinase involved in processing and activation of vacuolar precursor enzymes (Jones, 1984). In contrast to the immunological detection of accumulating proteinase yscB and carboxypeptidase yscY precursor molecules, no immunoreactive proteinase yscA molecules could be detected in *pep4-3* mutant cells (Mechler *et al.*, 1982b).

Recently the *PEP4* gene was found to code for proteinase yscA (Ammerer *et al.*, 1986; Woolford *et al.*, 1986). However, no data on the mechanism of activation and maturation of the vacuolar enzymes could be devised. Here we show that the *PEP4* gene is identical with the gene defined as *PRA1*, the structural gene of proteinase yscA, and we report on *in vivo* and *in vitro* studies shedding some light on the processing and activation mechanism of carboxypeptidase yscY.

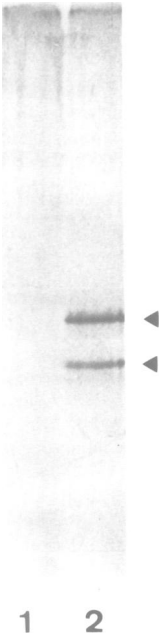

Fig. 1. *pra1-1* and *pep4-3* mutations are allelic. Protein in the heterozygous diploid strain *pra1-1/pep4-3* (**lane 1**) and in wild type strain *S288C* (**lane 2**) was labelled for 3 h at 30°C with [^{35}S]methionine. Labelling was done after shift of cells from high glucose to low glucose medium as described in Materials and methods. Immunoprecipitation of proteinase yscA immunoreactive protein with proteinase yscA antiserum, SDS–PAGE (10% gel) and fluorography was carried out as described by Mechler *et al.* (1982a,b). Upper arrow indicates position of pro-proteinase yscA (M_r = 52 000), lower arrow indicates mature proteinase yscA (M_r = 42 000).

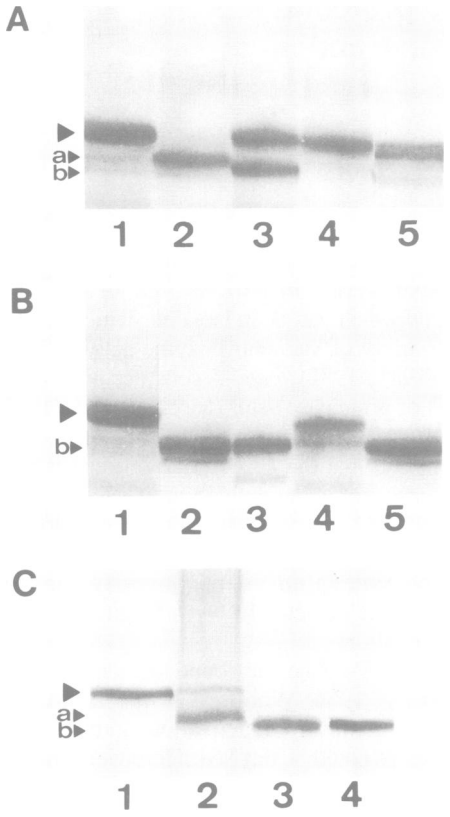

Fig. 2. Pro-carboxypeptidase yscY is processed by a proteinase yscA-triggered event and by proteinase yscB *in vitro*. Pro-carboxypeptidase yscY (20 μg) or carboxypeptidase yscY (0.5 μg) was incubated in 0.1 M Tris-maleate buffer, pH 5 for 3 h at 30°C in the presence of extracts of purified vacuoles, proteinase yscA or proteinase yscB. Incubation mixes were analyzed by SDS–PAGE (10% gel) followed by immunoblotting as described in Materials and methods. Upper arrow indicates position of pro-carboxypeptidase yscY. Lower arrows indicate position of processed carboxypeptidase $yscY^a$ (a) and carboxypeptidase $yscY^b$ (b). **(A)** Proteinase yscA-triggered processing of pro-carboxypeptidase yscY. **Lane 1:** pro-carboxypeptidase yscY (M_r = 67 000). **Lane 2:** pro-carboxypeptidase yscY incubated with extracts of purified vacuoles (50 μg protein) of strain *BYS232-31-4* (PRA^+ *prb1-1 prc1-1 cps1-1*) wild-type for proteinase yscA, defective in proteinase yscB. **Lane 3:** pro-carboxypeptidase yscY (M_r = 67 000) and mature, authentic carboxypeptidase yscY (M_r = 61 000) without addition. **Lane 4:** pro-carboxypeptidase yscY incubated as in lane 2 but with addition of 1 mM pepstatin. **Lane 5:** pro-carboxypeptidase yscY incubated with purified proteinase yscA (10 μg). **(B)** Proteinase yscB-triggered processing of pro-carboxypeptidase yscY. **Lane 1:** pro-carboxypeptidase yscY (M_r = 67 000). **Lane 2:** pro-carboxypeptidase yscY incubated with proteinase yscB (10 μg). **Lane 3:** mature, authentic carboxypeptidase yscY (M_r = 61 000). **Lane 4:** pro-carboxypeptidase yscY incubated as in lane 2, but with addition of proteinase yscB inhibitor I_2^B. **Lane 5:** pro-carboxypeptidase yscY incubated as in lane 2, but with addition of 1 mM pepstatin. **(C)** Processing of pro-carboxypeptidase yscY by a proteinase yscA-triggered event, followed by the action of proteinase yscB. **Lane 1:** pro-carboxypeptidase yscY (M_r = 67 000). **Lane 2:** pro-carboxypeptidase yscY incubated with vacuoles lacking proteinase yscB but containing intact proteinase yscA. **Lane 3:** pro-carboxypeptidase yscY incubated as in lane 2. After 3 h of incubation proteinase yscB was added and incubation was continued for another 3 h at 30°C. **Lane 4:** mature, authentic carboxypeptidase yscY.

Results

We had isolated proteinase yscA mutants and by this had avoided selecting any mutant exhibiting the pleiotropic *pep4* phenotype. All the proteinase yscA mutants which we studied were completely devoid of the hemoglobin splitting activity of proteinase yscA but exhibited proteinase yscB and carboxypeptidase yscY activities (Mechler and Wolf, 1981; Mechler *et al.*, 1982b). The mutations with the genotype designation *pra1-1*, *pra1-2*, *pra1-3* and *pra1-4* were shown to reside in the structural gene of proteinase yscA. Two of the mutants, *HbI* (*pra1-1*) and *HbIV* (*pra1-4*), were completely devoid of proteinase yscA antigenic material (Mechler *et al.*, 1982b).

Thus, we were faced with two kinds of mutants; on one hand *pra1-1* and *pra1-4* and on the other hand *pep4-3*, which exhibit the same phenotype; namely, lack of proteinase yscA activity against hemoglobin and being without antigenic proteinase yscA protein but differing in that *pra*-mutants have active proteinase yscB and carboxypeptidase yscY while the *pep4-3* mutant has not.

To see whether the *pra1-1* and *pep4-3* mutations were in the same gene or in different genes, we constructed diploid strains heterozygous for both mutations. Extracts of such a heterozygous strain did not show any proteinase yscA activity against the standard substrate hemoglobin nor was any proteinase yscA antigenic material found (Figure 1, lane 1). When this diploid strain was sporulated, none of the spores derived showed any proteinase yscA activity (not shown). These results strongly suggest that *pra1* and *pep4* are mutations in the same gene, which is the structural gene of proteinase yscA. These observations are strongly supported by recent results of two other research groups. Sequencing of the DNA stretches coding for proteinase yscA and for the *pep4-3* gene product resulted in the same nucleotide sequence (Ammerer *et al.*, 1986; Woolford *et al.*, 1986).

Thus, as *pep4-3* mutants are maturation deficient, it seems obvious that proteinase yscA is involved in the processing event of vacuolar proteinase precursors. This is underlined by the fact that introduction of the proteinase yscA structural gene into the *pep4-3* mutant strain leads to wild-type phenotype concerning proteinase yscA, proteinase yscB and carboxypeptidase yscY activities (Rothman *et al.*, 1986). The different phenotype of *pra1-1* and *pep4-3* mutants concerning the activation of vacuolar enzymes might, at first glance, be explained by the fact that the *pra1-1* mutant is somewhat leaky, leading to an intracellular level of proteinase yscA sufficient for activation of the precursor proteins to occur but not sufficient to lead to normal protein turnover and to be detectable at the activity and protein level *in vitro* (D.H.Wolf and U.Teichert, unpublished; Mechler and Wolf, 1982b).

We therefore tested the hypothesis that proteinase yscA was the activating principle by incubating proteinase yscA with purified, inactive carboxypeptidase yscY precursor. Surprisingly, neither commercially available proteinase yscA (Figure 2A, lane 5) nor purified proteinase yscA isolated from intact vacuoles was able to process and activate the precursor enzyme (Table I). Depending on the concentration of proteinase yscA in the incubation mixtures, lower mol. wt forms of pro-carboxypeptidase yscY were found which were, however, inactive (not shown). This indicates that even though proteinase yscA is a component of the maturating system of vacuolar enzymes, it is not sufficient for maturation to occur. We thus expected to obtain further information on the processing event by analyzing the *pra1* mutants isolated previously (Mechler and Wolf, 1981; Mechler *et al.*, 1982b).

As expected from the fact that all four proteinase yscA mutants analyzed exhibit carboxypeptidase yscY activity (Mechler and Wolf, 1981), they also contain mature carboxypeptidase yscY. As an example, Figure 3A shows maturation of the enzyme in the *pra1-1* mutant.

As mentioned before, lack of proteinase yscB in a mutant (*prb1-1*) (Wolf and Ehmann, 1978,1979) does not affect pro-

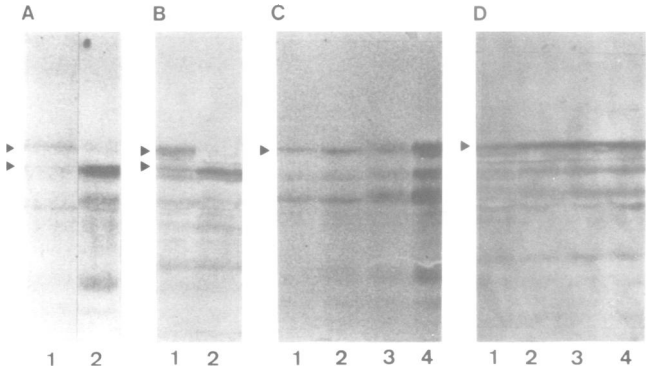

Fig. 3. Pro-carboxypeptidase yscY is processed by proteinase yscA and proteinase yscB-triggered events *in vivo*. Protein was labelled with [^{35}S]methionine in different mutant strains. Growth of cells, pulse-chase labelling, immunoprecipitation of carboxypeptidase yscY immunoreactive protein with carboxypeptidase yscY antiserum, SDS–PAGE (10% gel) and fluorography were done as outlined in Mechler *et al.* (1982a,b). Upper arrow indicates position of pro-carboxypeptidase yscY, lower arrow indicates position of matured carboxypeptidase yscY. **(A)** proteinase yscA-deficient mutant strain *HbI-2-11A* (*pra1-1* PRB$^+$). **Lane 1:** 20-min pulse, **lane 2:** 120-min chase. **(B)** proteinase yscB-deficient strain *UT-9B* (PRA$^+$ *prb1-1*). **Lane 1:** 20-min pulse, **lane 2:** 120-min chase. **(C)** proteinase yscA-deficient mutant strain *20B-12* (*pep4-3*). **Lane 1:** 20-min pulse, **lane 2:** 20-min chase, **lane 3:** 60-min chase, **lane 4:** 120-min chase. **(D)** proteinase yscA- and proteinase yscB-deficient strain *UT-9D* (*pra1-1 prb1-1*). **Lane 1:** 20-min pulse, **lane 2:** 20-min chase, **lane 3:** 60-min chase, **lane 4:** 120-min chase.

Table I. *In vitro* activation of pro-carboxypeptidase yscY by proteinase yscA- or proteinase yscB-triggered reactions

Incubation of pro-carboxypeptidase yscY with	Specific activity
First set of experiments:	
No addition	0.02
Proteinase yscA	0.02
Vacuoles of strain *BYS232-31-4* (PRA$^+$ *prb1-1*)	0.53
Vacuoles of strain *BYS232-31-4* (PRA$^+$ *prb1-1*) + pepstatin	0.01
Proteinase yscB	0.54
Proteinase yscB + I_2^B	0.12
Second set of experiments:	
Proteinase yscA	0.06
Vacuoles of strain *20B-12* (*pep4-3*)	0.05
Vacuoles of strain *ABYS66* (*pra1-1 prb1-1*)	0.01
Proteinase yscA + vacuoles of strain *20B-12* (*pep4-3*)	0.39
Proteinase yscA + vacuoles of strain *ABYS66* (*pra1-1 prb1-1*)	0.44
Proteinase yscA + NaCl (1 M)	0.21
Proteinase yscA + polyphosphoric acid mixture (0.25 M)	0.24
Proteinase yscA + $Na_{47}P_{45}O_{136}$ (0.05 M)	0.34

Pro-carboxypeptidase yscY (20 μg) was incubated with additions as outlined in Materials and methods. Additions were: proteinase yscA, 10 μg; proteinase yscB, 10 μg; vacuoles of strains indicated, 50 μg of protein; pepstatin (0.66 mM); proteinase yscB inhibitor I_2^B (0.93×10^3 $\Delta E_{520\ nm}$/min × ml) (Betz *et al.*, 1974). Specific activity is given as activity of processed pro-carboxypeptidase yscY/mg of incubated pro-carboxypeptidase yscY × min of test.

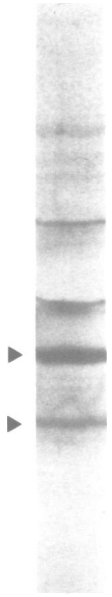

Fig. 4. Mutant strain HbI-2-11A (*pra1-1* PRB$^+$) contains mature proteinase yscB. Cells were labelled for 20 min at 30°C with [^{35}S]methionine. Immunoprecipitation of immunoreactive protein with proteinase yscB antiserum, SDS–PAGE (10% gel) and fluorography was done as outlined in Mechler *et al.* (1982a,b). Upper arrow indicates position of pro-proteinase yscB (M_r = 42 000), lower arrow indicates position of mature proteinase yscB (M_r = 33 000).

cessing of pro-carboxypeptidase yscY (Figure 3B). Further insight into the processing event *in vivo* was gained by combining the *pra1-1* with the *prb1-1* mutation (Figure 3D): the double mutant was unable to process and activate pro-carboxypeptidase yscY. As seen in the case of the *pep4-3* mutant (Figure 3C), the double mutant accumulated the pro-form of the enzyme. (Bands not marked with arrows are not due to matured carboxypeptidase yscY. This cross-reacting material is of lower mol. wt and considered to be due to degradation products of the enzyme.) Thus, in the *pra1-1* mutant (Figure 3A) processing of pro-carboxypeptidase yscY must have occurred via proteinase yscB, whereas in the strain carrying the *prb1-1* mutation processing must have occurred via proteinase yscA (Figure 3B). This clearly indicates that pro-carboxypeptidase yscY can be processed *in vivo* by a proteinase yscB- as well as by a proteinase yscA-triggered event.

In contrast to the *pep4-3* mutant strain (Mechler *et al.*, 1982b), the *pra1-1* mutant has retained the ability to process pro-proteinase yscB (Figure 4). Thus, the differences in processing pro-carboxypeptidase yscY in the proteinase yscA mutant *pra1-1* isolated by us and the *pep4-3* mutant isolated by Jones (1977) must reside in the different abilities of these mutants to process proteinase yscB.

As shown before (Figure 2), the set-up of an *in vitro* system to explain processing by using commercial or purified proteinase yscA from vacuoles was unsuccessful. As had been demonstrated earlier (Hasilik and Tanner, 1978), proteinase yscB is able to process pro-carboxypeptidase yscY to its apparently authentic mol. wt form *in vitro*. This is again demonstrated by showing that *in vitro* processing of pro-carboxypeptidase yscY can be inhibited by the specific proteinase yscB inhibitors I_2^B (Figure 2B, lanes 2 and 4) and chymostatin (not shown).

To obtain further insight into the role of proteinase yscA in the processing event, we isolated vacuoles of a strain devoid of proteinase yscB that was additionally defective in carboxypeptidase yscY and yscS activities.

In contrast to purified proteinase yscA from the same vacuoles, the whole vacuolar fraction from the proteinase yscB mutant strain is able to process pro-carboxypeptidase yscY and to activate the enzyme (Figure 2A, lane 2; Table I). Optimum pH of the ac-

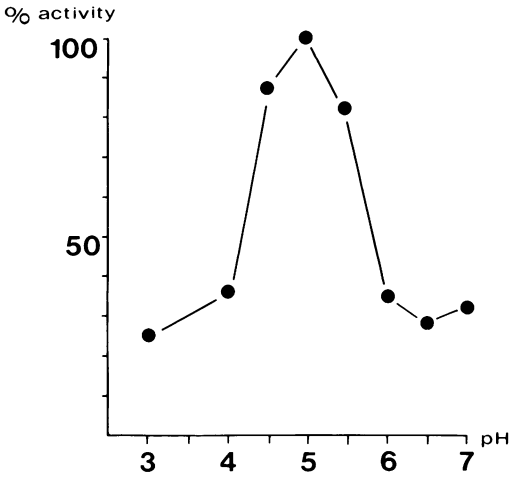

Fig. 5. pH optimum of *in vitro* activation of pro-carboxypeptidase yscY. pH optimum of the activation reaction was determined using 0.1 M Tris−maleate buffer adjusted to the respective pH values with 1 M NaOH or 1 M HCl. Measurement was done using 20 μg pro-carboxypeptidase yscY and 37 μg of vacuoles of strain *BYS 232-31-4* in a volume of 0.03 ml. Incubation was done for 4 h at 30°C. Carboxypeptidase yscY activity was tested as outlined in Materials and methods.

tivation process is pH 5 (Figure 5). However, processing of the carboxypeptidase yscY precursor by the vacuolar fraction of this strain results in a processed protein of somewhat higher mol. wt as compared to authentic, mature carboxypeptidase yscY (compare Figure 2A, lanes 2 and 3). Pepstatin, an inhibitor of acid proteinases and specifically blocking proteinase yscA activity, completely abolishes the maturation and activation process (Figure 2A, lane 4; Table I). These experiments indicate that proteinase yscA is involved in the processing and activation event.

The processed form of carboxypeptidase yscY of intermediate mol. wt resulting from proteinase yscA action in the vacuoles can be transferred by addition of proteinase yscB *in vitro* into mature carboxypeptidase yscY protein of apparently authentic mol. wt (Figure 3C).

On the basis of the finding that the vacuolar fraction containing proteinase yscA but deficient in proteinase yscB leads to maturation of pro-carboxypeptidase yscY to a species of somewhat higher mol. wt as compared to the enzyme processed by proteinase yscB, we analyzed the mol. wt of matured carboxypeptidase yscY in the *pra1-1* mutant and in the *prb1-1* mutant in more detail. As can be seen from Figure 6, the mol. wt of processed carboxypeptidase yscY is also different *in vivo*.

As different yeast strains seem to glycosylate carboxypeptidase yscY to slightly different extents leading to carboxypeptidase yscY molecules of different mol. wt (not shown), we confirmed that the mol. wt differences of carboxypeptidase yscY in the *pra1-1* mutant and in the *prb1-1* mutant are due to differences in the mol. wt of the protein moiety of the enzyme. Isolation of carboxypeptidase yscY from both strains after treatment of cells with tunicamycin, an inhibitor of glycosylation of the enzyme, leads to the same mol. wt differences in carboxypeptidase yscY as in untreated *pra1-1* and *prb1-1* mutant cells (not shown).

As expected from the *in vivo* phenotype of the *pep4-3* mutant strain, extracts of vacuoles of this mutant do not show any processing of pro-carboxypeptidase yscY as had been also found for the *pra1-1 prb1-1 prc1-1 cps1-3* mutant strain (Figure 7, lanes 4 and 6; Table I). However, addition of vacuolar extracts of either one of these two mutants to purified proteinase yscA completely

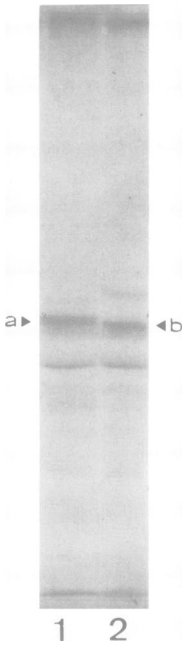

Fig. 6. Maturation of pro-carboxypeptidase yscY by proteinase yscA and proteinase yscB-triggered events *in vivo* leads to mature carboxypeptidase yscY species of different mol. wt. Strain *UT-9B* (PRA$^+$ *prb1-1*) **(lane 1)** and strain *UT-9A* (*pra1-1* PRB$^+$) **(lane 2)** were labelled for 1 h with [^{35}S]methionine as described in Materials and methods. Labelling was done after shift of cells from high glucose to low glucose medium. Immunoprecipitation of carboxypeptidase yscY immunoreactive protein with carboxypeptidase yscY antiserum, SDS−PAGE (10% gel) and fluorography were performed as described by Mechler *et al.* (1982a,b).

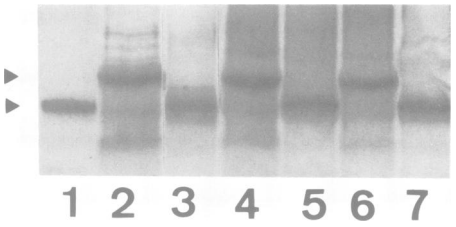

Fig. 7. Processing of pro-carboxypeptidase yscY by proteinase yscA is dependent on a vacuolar component. Pro-carboxypeptidase yscY (20 μg) or carboxypeptidase yscY (0.5 μg) was incubated in 0.1 M Tris−maleate buffer, pH 5 for 3 h at 30°C in the presence or absence of proteinase yscA (1.8 μg) and vacuolar extracts (50 μg protein). All incubation mixtures contained chymostatin (4 μg/ml). Incubation mixes were analyzed by SDS−PAGE (10% gel) followed by immunoblotting as described in Materials and methods. **Lane 1:** mature, authentic carboxypeptidase yscY (M_2 = 61 000). **Lane 2:** pro-carboxypeptidase yscY (M_r = 67 000). **Lane 3:** pro-carboxypeptidase yscY incubated with extracts of purified vacuoles of strain *BYS232-31-4* (PRA$^+$ *prb1-1 prc1-1 cps1-3*) wild-type for proteinase yscA, defective in proteinase yscB. **Lane 4:** pro-carboxypeptidase yscY incubated with extracts of purified vacuoles of strain *20B-12* (*pep4-3*). **Lane 5:** pro-carboxypeptidase yscY incubated as in lane 4, but with addition of purified proteinase yscA. **Lane 6:** pro-carboxypeptidase yscY incubated with extracts of purified vacuoles of strain *ABYS66* (*pra1-1 prb1-1 prc1-1 cps1-3*) defective in proteinase yscA and proteinase yscB activities. **Lane 7:** pro-carboxypeptidase yscY incubated as in lane 6 but with addition of purified proteinase yscA.

restores processing and activation of pro-carboxypeptidase yscY, yielding the same pattern as found with vacuoles lacking proteinase yscB but containing intact proteinase yscA (Figure 7, lanes 3, 5 and 7; Table I). This clearly indicates that vacuoles contain a component which acts in concert with proteinase yscA to yield processed and active carboxypeptidase yscY. *pep4-3* mutant

vacuoles and *pra1-1 prb1-1 prc1-1 cps1-3* mutant vacuoles seem to be identical in this component. No additional vacuolar enzyme dependent on the activation by proteinase yscA and involved in the maturation cascade could be resolved as yet *in vitro*. This does not exclude the presence of such an enzyme *in vivo*.

In a search for a component triggering activation and processing of pro-carboxypeptidase yscY in concert with proteinase yscA we found that addition of high concentrations of NaCl to purified proteinase yscA could substitute for the vacuolar extracts in activating pro-carboxypeptidase yscY *in vitro* (Table I). This led us to assume that some vacuolar salt component might be the additional factor enabling proteinase yscA to mature pro-carboxypeptidase yscY. A characteristic vacuolar component is polyphosphate (Urech *et al.*, 1978; Okorokov *et al.*, 1980). Addition of a polyphosphoric acid mixture or sodium polyphosphate of defined chain length to purified proteinase yscA led to activation (Table I) and maturation (not shown) of pro-carboxypeptidase yscY. Whether or not the processing of pro-carboxypeptidase yscY that can be observed in the presence of polyphosphate is identical to the maturation exerted by the inclusion of vacuoles cannot be decided by the method employed. It can also not be decided if polyphosphate or some other salt component is the component acting in concert with proteinase yscA *in vivo* in activating and processing pro-carboxypeptidase yscY. The elucidation of the character of the component rests on future studies. Maturation defective mutants with lesions in genes different from the *PRA1/PEP4* gene should give answer. Such studies are underway.

Discussion

Our studies demonstrate that maturation of pro-carboxypeptidase yscY depends on the action of proteinase yscA and proteinase yscB. The complete lack of processing and activation of pro-carboxypeptidase yscY in the *pep4-3* mutant carrying a mutated proteinase yscA structural gene indicates that processing is dependent on a product of the proteinase yscA gene. Experiments on the *pra1-1* mutant carrying also a structural gene mutation in the proteinase yscA gene but showing processing of pro-carboxypeptidase yscY (Figure 3A) suggest that abolishing proteinase yscA activity is not sufficient to block maturation and activation. The argument that the *pra1-1* mutant was somewhat leaky in proteinase yscA activity allowing processing of pro-carboxypeptidase yscY to occur can be excluded by the fact that maturation of pro-carboxypeptidase yscY is completely blocked when a mutation in the structural gene of proteinase yscB (*prb1-1*) is introduced into the *pra1-1* mutant (Figure 3D). Thus, the capability of the *pra1-1* mutant to process and activate pro-carboxypeptidase yscY must reside in the activity of proteinase yscB, indicating the involvement of this enzyme in the maturation process of carboxypeptidase yscY. The involvement of proteinase yscB in the processing event is in addition documented by the appearance of a processed form of carboxypeptidase yscY of intermediate size in a proteinase yscB mutant (Figure 6) and by generation of such a form *in vitro*, when vacuole extracts of the proteinase yscB mutant are used to mimic processing (Figure 2).

Complete loss of maturating activity of pro-carboxypeptidase yscY in vacuolar extracts of mutants lacking both endoproteinases yscA and yscB activities (*ABYS1*) shows again direct involvement of a product of the proteinase yscA gene in the processing event (Figure 7). This processing activity is also lost in vacuolar extracts of the *pep4-3* mutant (Figure 7). This shows that both mutant strains (*pra1-1* and *pep4-3*) have the same defect in abolishing proteinase yscA-triggered processing of pro-carboxypeptidase yscY.

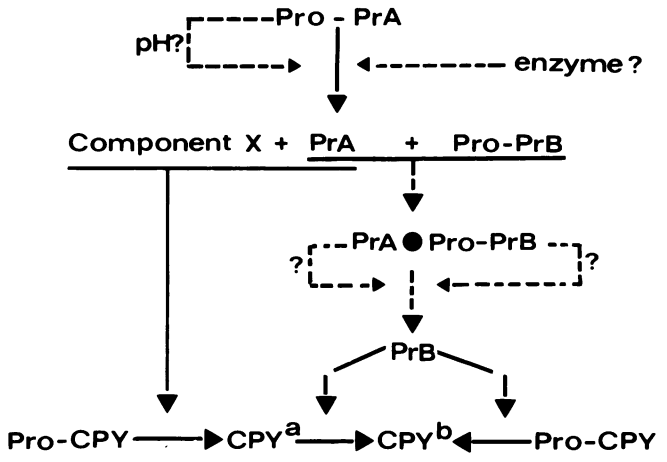

Fig. 8. Putative model of maturation reactions yielding active carboxypeptidase yscY. (Pro-) PrA = (Pro-) proteinase yscA, (Pro-) PrB = (Pro-) proteinase yscB, (Pro-) CPY = (Pro-) carboxypeptidase yscY. Dotted arrows: hypothetical reactions. Non-dotted arrows: reactions based on experimental results.

In contrast to vacuolar extracts of strains wild-type for proteinase yscA activity, purified proteinase yscA is unable to process and activate pro-carboxypeptidase yscY (Figure 2). This indicates an additional component present in vacuolar extracts, necessary for processing and activation to occur, which is lost during proteinase yscA purification. This conclusion is underlined by the fact that addition of extracts from vacuoles of mutant strain *pep4-3* or strain *ABYS1* lacking both endoproteinases yscA and yscB to purified proteinase yscA restores processing of pro-carboxypeptidase yscY (Figure 7). Whether this component is actually polyphosphate (Table I) or an enzyme dependent on the activation by proteinase yscA has to be proven in future studies.

Our *pra1-1* mutant shows processing of pro-proteinase yscB (Figure 4). In contrast, processing of proteinase yscB seems to be completely blocked in the *pep4-3* mutant (Mechler *et al.*, 1982b). As *pra1-1* and *pep4-3* are mutations in the structural gene of proteinase yscA, some component of the proteinase yscA gene must be responsible for the activation of proteinase yscB. *pra1* mutants and the *pep4-3* mutant must differ in the activity of this component. The data discussed above would fit the model proposed (Figure 8).

The processing and activation cascade might be triggered by the self-activation of pro-proteinase yscA or by activation of proteinase yscA catalyzed by an as yet unknown enzyme. This view is supported by the observation that a mutant lacking proteinase yscB and carboxypeptidase yscY and yscS activities shows normal activation of proteinase yscA (not shown). Proteinase yscA in concert with some vacuolar component (component X) converts pro-carboxypeptidase yscY to an active intermediate mol. wt form (CPY^a). Mature proteinase yscB converts pro-carboxypeptidase yscY to an equally active enzyme with the apparently authentic mol. wt of mature carboxypeptidase yscY (CPY^b). The *in vitro* experiments indicate that proteinase yscB is also able to convert the proteinase yscA-generated intermediate mol. wt form of carboxypeptidase yscY (CPY^a) into the apparently authentic, mature form (CPY^b).

If proteinase yscA were the catalyst processing pro-carboxypeptidase yscY, the fact that this catalysis does not lead to authentic carboxypeptidase yscY can be explained by comparing the

substrate specificity of proteinase yscA elucidated so far and the peptide bond to be cleaved in the pro-carboxypeptidase yscY molecule to yield the authentic, mature species. Proteinase yscA catalyzes the cleavage of peptide bonds between two hydrophobic residues (Dreyer *et al.*, 1985), whereas the site to be cleaved to yield authentic, mature carboxypeptidase yscY is an Asn−Lys bond (Valls *et al.*, 1987).

Little is known about the processing mechanism of pro-proteinase yscB. The lack of active and mature proteinase yscB in *pep4-3* mutants clearly indicates that the proenzyme is not able to process itself without at least part of the product coded by the proteinase yscA gene. Some hypotheses may be proposed. Processing might simply occur via hydrolysis of pro-proteinase yscB by an active proteinase yscA yielding the active, mature product. This is, however, hard to reconcile with the fact that in contrast to carboxypeptidase yscY, proteinase yscB activity shows gene dosage which is dependent on the copy number of the *PEP4-3* gene (Jones *et al.*, 1982; Woolford *et al.*, 1986). One possibility to explain this might be a stable 1:1 complex formation between proteinase yscA and pro-proteinase yscB followed by processing of the complex. Stable complex formation between proteinase yscA and proteinase yscB has been demonstrated (Hinze *et al.*, 1975). The *pra1-1* mutant is able to process pro-proteinase yscB indicating that, despite its proteinase yscA antigen negative phenotype, some proteinase yscA product must be made. As the *pra1-1* mutation completely abolishes catalysis of carboxypeptidase yscY processing we wonder whether the *pra1-1* gene product is able to catalyze pro-proteinase yscB maturation. We favor the idea instead that the *pra1-1* gene product might have retained those sequences necessary for complex formation with pro-proteinase yscB which initiates a self-maturation of proteinase yscB in the complex. Future studies must prove or disprove this model.

Materials and methods

Yeast strains

Wild-type strain was *S288C* (α) (Mechler *et al.*, 1982a). Proteinase yscA-deficient mutants used were *HbI-2-11A* (*a pra1-1 ade*), *UT-9A* (*a pra1-1 ade his7 lys2*) (Mechler *et al.*, 1982b; B.Mechler, U.Teichert and D.H.Wolf, unpublished), and strain *20B-12* (*α pep4-3 trp1*) (Jones, 1977). Proteinase yscB-deficient mutants used were: *HP232-2B* (*α prb1-1 ade2 ura1 his7 lys2*) (Wolf and Ehmann, 1979) and *UT-9B* (*a prb1-1 ade*) (B.Mechler, U.Teichert and D.H.Wolf, unpublished). Proteinase yscA- and proteinase yscB-deficient double mutant strains were *UT-9D* (*α pra1-1 prb1-1 ade ura1 his7*) (B.Mechler, U.Teichert and D.H.Wolf, unpublished), and the transformed strain *PEPB-3* (*pep4-3 prb1-1 leu2* PRC: pTSY1) which originated from a cross of strain *SF757-1D* (*a pep4-3 leu2* PRC: pTSY1) (Stevens *et al.*, 1986) and strain *HM1* (*α prb1-1 leu2*) (H.Müller and D.H.Wolf, unpublished). Diploids were isolated, subjected to sporulation and tetrads were dissected as outlined by Fink (1970). Strains containing the pro-carboxypeptidase yscY-containing plasmid pTSY1 as deduced from their growth behavior on plates without supplemented leucine and being devoid of proteinase yscB as measured with Azocoll in the presence of 0.25% SDS were picked. Proteinase yscB-, carboxypeptidase yscY- and carboxypeptidase yscS-deficient triple mutant was *BYS232-31-4* (*a prb1-1 prc1-1 cps1-3 ade ura1 leu2*) (Achstetter and Wolf, 1985b). Proteinase yscA-, proteinase yscB-, carboxypeptidase yscY- and carboxypeptidase yscS-deficient quadruple mutant was strain *ABYS66* (*a pra1-1 prb1-1 prc1-1 cps1-3 ade*) isolated from the same cross as strain *ABYS1* described in Achstetter *et al.* (1984). A heterozygous diploid *pep4-3/pra1-1* was obtained from a cross of strain *20B-12* × *HbI-2-11A*.

Media

Complete medium (YPD) contained 1% yeast extract, 2% peptone and 2% glucose. Mineral medium contained 0.67% yeast nitrogen base without amino acids and 2% glucose supplemented with the compounds required by the auxotrophy of strains. High glucose medium was mineral medium with 5% glucose, low glucose medium was mineral medium with 0.3% glucose. Sporulation medium contained 1% potassium acetate, 0.05% glucose and 0.1% yeast extract. When these media were used as plate media, they contained 2% agar.

Enzymes and inhibitors

Proteinase yscA was either obtained from Sigma or purified from vacuolar extracts of strain *BYS232-31-4* by gel filtration on Sephadex G-75. Source of proteinase yscB was either a proteinase yscA preparation of Sigma (batch no. 39C-39291), which contained the enzyme in substantial amounts, or purified proteinase yscB obtained from H.Hoffschulte, Freiburg (Kominami *et al.*, 1981). Carboxypeptidase yscY, chymostatin and pepstatin were obtained from the Peptide Institute, Osaka, Japan. Proteinase yscB inhibitor I_2^B was a generous gift of Dr P.Bünning, Freiburg.

Immunsera

Immunsera against proteinase yscA, proteinase yscB and carboxypeptidase yscY had been characterized previously (Mechler *et al.*, 1982a,b).

Enzyme assays

Proteinase yscA was tested against acid hemoglobin as substrate and proteinase yscB was assayed with Azocoll as substrate as described by Saheki and Holzer (1974). Carboxypeptidase yscY was measured using benzoyl-tyrosine-4-nitroanilide as a substrate as described by Aibara *et al.* (1971). Pro-carboxypeptidase yscY was activated with trypsin (1 mg/ml) for 60 min at 37°C. Subsequently carboxypeptidase yscY activity was measured in the presence of chymostatin (0.14 μg/ml test volume). α-Mannosidase was tested with p-nitrophenyl-α-D-mannopyranoside as described by Van der Wilden *et al.* (1973).

Isolation of pro-carboxypeptidase yscY

Strain *PEPB-3* carrying a high copy plasmid with pro-carboxypeptidase yscY and harboring chromosomal *pep4-3* and *prb1-1* mutations was grown on mineral medium supplemented with histidine, lysine, adenine and uracil for 36 h at 30°C. All subsequent steps were done at 4°C. Cells were harvested by centrifugation, washed with 0.1 M Tris−HCl buffer, pH 7.2, resuspended in this buffer (cell weight:buffer volume, 1:1) and broken in a French Pressure cell at 20 000 lb/in.^{2}. Crude extracts were centrifuged for 10 min at 18 000 r.p.m. The supernatant was recentrifuged for 45 min at 35 000 r.p.m. The supernatant was dialyzed against 0.02 M Tris−HCl buffer. Proteins of the dialyzed extract (600 mg protein) were separated using DEAE−Sepharose CL-6B ion-exchange chromatography (gel bed 14 ml). Separation was done using a linear salt gradient of 0−400 mM NaCl in 0.02 M Tris−HCl buffer, pH 7.2. Active fractions were pooled and dialyzed against 1 mM potassium phosphate buffer, pH 7. The dialyzed extract (14 mg protein) was applied on a hydroxy-apatite column (Ultrogel HA, LKB; 42 ml gel bed) and the proteins were eluted with a linear gradient of 1−100 mM potassium phosphate, pH 7. Active fractions were pooled and concentrated with polyethylene glycol. The concentrated material was dialyzed against 0.02 M Tris−HCl buffer, pH 7.2. Purification of the pro-enzyme ranged from 40- to 70-fold.

Isolation of vacuoles

Cells were grown in YPD medium for 36 h at 30°C. Spheroplast formation, lysis of spheroplasts with DEAE−dextran and purification was performed as described by Wiemken *et al.* (1979) with the following modifications. Pre-incubation of cells was carried out in 0.6 M sorbitol, 25 mM piperazin-*N*,*N*'-bis(2-ethane sulfonic acid) adjusted with 2-amino-2-methyl 1,3-propanediol to pH 6.8 and 0.14 M cysteamine/HCl. Spheroplast formation was done with Zymolyase 20 000 (5 mg/g of cells). Enrichment of vacuoles was 20- to 30-fold.

Biosynthesis of vacuolar enzymes in vivo

Labelling of cells was performed with [^{35}S]methionine (300 μCi/ml of cell suspension). Labelling, immunoprecipitation, SDS−PAGE and fluorography were done as outlined by Mechler *et al.* (1982a,b).

Processing of pro-carboxypeptidase yscY in vitro

Pro-carboxypeptidase yscY was incubated for 3 h at 30°C as described in the figure legends and the incubation mixtures were divided into two parts. One part was tested biochemically for carboxypeptidase yscY activity, the other part was subjected to trichloroacetic acid precipitation (5%) followed by SDS−PAGE and electroimmunoblotting. Electroimmunoblotting to nitrocellulose was done as described by Towbin *et al.* (1979) with the following modifications. Blotting buffer was 0.02 M Tris−0.15 M glycine. After transfer of proteins and precoating of the nitrocellulose sheets with bovine serum albumin, an additional coating with gelatine (0.25%) was performed. After incubation of the nitrocellulose sheets with carboxypeptidase yscY antiserum and peroxidase-conjugated goat anti-rabbit antibodies, the color reaction at the sites of antigenic protein was carried out with 4-chloro-1-naphthol.

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