Lysosomal (Vacuolar) Proteinases of Yeast Are Essential Catalysts for Protein Degradation, Differentiation, and Cell Survival*

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Mutants deficient in the vacuolar (lysosomal) endopeptidases proteinase yscA and proteinase yscB of the yeast Saccharomyces cerevisiae exhibit a drastically reduced protein degradation rate under nutritional stress conditions. The differentiation process of sporulation is considerably disturbed by the absence of the two endopeptidases. Also under vegetative growth conditions and under conditions of false protein synthesis, the two vacuolar endopeptidases exhibit some effect on protein degradation, which is, however, much less pronounced as found under starvation conditions. Proteinase yscA deficiency leads to rapid cell death when glucose-grown cells starve for nitrogen or other nutrients. Whereas overall protein degradation is affected in the endopeptidase mutants, degradation of two distinct false proteins analyzed is not altered in the absence of proteinase yscA and proteinase yscB. Also catabolite inactivation and degradation of fructose-1,6-bisphosphatase is not affected to a greater extent in the endopeptidase-deficient strains.

The ease of biochemical, genetic, and molecular biological handling has made the yeast Saccharomyces cerevisiae an excellent tool to study cellular control in the eukaryotic cell. The exact nature of the intracellular functions of proteolytic enzymes has become a central issue in recent years. In the yeast S. cerevisiae a multitude of proteolytic enzymes have been found (1, 2). Functions range from specific protein processing (1–4) to general protein degradation (1, 2, 5, 6). In cases studied in detail, the yeast enzymes seem to be the genuine prototypes of their higher eukaryotic cell counterparts (7–14). The lysosome has been found to be a compartment of protein degradation in mammalian cells (15). The vacuole has been proposed to be the lysosome of the yeast cell (16, 17).

A variety of hydrolases, among them two endopeptidases, proteinase yscA and proteinase yscB, and five exopeptidases, carboxypeptidases yscY and yscS, aminopeptidases yscI and yscCo as well as dipetidyl aminopeptidase yscV, have been shown to be associated with this cellular organelle (1, 2, 5, 6). In order to study the function of the yeast lysosome and the function of its proteolytic enzyme equipment, we (18–23) and others (24–26) have isolated and characterized mutants of the vacuolar proteolytic enzymes. Here we report further on the impact of the two vacuolar endopeptidases, proteinase yscA and proteinase yscB, on cellular physiology.

**MATERIALS AND METHODS**

**RESULTS**

Impact of the Vacuolar Endopeptidases Proteinase yscA and Proteinase yscB on Protein Degradation in Starving and Growing Cells—The level of the two vacuolar proteinases yscA and yscB is rather low under conditions of vegetative growth on glucose (39) and increases considerably under conditions of poor carbon supply and nitrogen starvation (29, 39, 40). When diploid MATα/MATα yeast cells are transferred to such nutritional stress conditions (acetate medium, free of nitrogen), they undergo the differentiation process of sporulation (41). We had isolated structural gene mutants deficient in the activities of proteinase yscA (21) and proteinase yscB (11, 19, 20, 22, 27) and had shown that strains carrying a single proteinase mutation were affected in protein degradation under starvation conditions and in sporulation (20–22). We extended these studies in analyzing double proteinase-deficient mutants under nutritional stress and under vegetative growth conditions. As can be seen in Fig. 1, transfer of proteinase yscA (pral-1) or proteinase yscB (prbl-1)-deficient diploid cells onto nitrogen-free acetate (sporulation) medium yields a considerably decreased protein degradation rate in both mutants. While the degradation rate in wild type cells is 3.3%/h within the first 8 h after transfer to the sporulation medium, it is only about 1%/h in proteinase yscA mutants and about 1.16%/h in proteinase yscB mutants within the time interval of the first 8 h after transfer onto this medium. These degradation rates in the mutants amount to only 31% (proteinase yscA mutant) and 35% (proteinase yscB mutant) of the wild type level. Double mutants deficient in the activities of proteinase yscA and proteinase yscB (pral-1 prbl-1) exhibit only a degradation rate of 0.46%/h during the first 8 h on sporulation medium under the same conditions, which amounts to 14% of wild type (Fig. 1). This dramatic decrease of protein degradation in vivo due to absence of the two vacuolar endopeptidases, proteinase yscA and proteinase yscB, coincides also with in vitro measurements. Crude extracts of proteinase yscA and proteinase yscB double mutants have lost 97% of [3H]methylcasein degradation capacity and 86% of their capacity to degrade [3H]leucine-labeled yeast protein (not shown).

1 Portions of this paper (including Materials and Methods and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
pep4-3
ped-3
boxypeptidase yscY (42). Recently, it has been shown that processes carboxypeptidase yscY, whereas the under sporulation conditions (43). Proteinase yscA, protein-3 yscA turned out to be the trigger of the maturation event of value of 30-38% as compared with wild type was observed the proteolytic maturation of proteinase yscB (27) and car-3 of the 42-kDa precursor of proteinase yscB and of the precursor is unable to do so (10). Astonishingly, even though the proteinase yscB activities and not in part due to the additional absence of carboxypeptidase yscY which is contained in this mutant as inactive precursor. For this reason we measured protein degradation in a proteinase yscA-proteinase yscB double mutant (pral-1 prbl-1) carrying another mutated proteinase yscA allele pral-2, which also in the absence of proteinase yscB is able to at least partly activate carboxypeptidase yscY (not shown). A residual protein degradation rate of 10% of wild type was found, indicating that carboxypeptidase yscY was not responsible for the drop in protein degrad-3ving capacity in pral-1 prbl-1 mutants. Carboxypeptidase yscY acts most likely on the peptides which are generated by proteinase yscA and proteinase yscB.

Vegetatively growing cells contain considerably lower levels of proteinase yscA and proteinase yscB as do starving cells (39). We investigated whether the two vacuolar endopepti-3ases also take part in protein degradation in actively growing cells. Protein degradation in wild type and in proteinase yscA-proti-3inase yscB (pral-1 prbl-1) double mutant strains growing logarithmically on glucose and ammonium sulfate were compared (Fig. 2A). Cells were radioactively labeled for 0.5 h before transfer onto fresh medium. Degradation during the first hour was rapid and most likely due to proteins of short half-life, whereas thereafter the class of proteins with a longer half-life is determined (44). Under these conditions 5.3% of protein is degraded in wild type cells during the first hour after transfer, whereas 3.5% is degraded in the double mutant defective in proteinase yscA and proteinase yscB. The average degradation rate, after the first hour of transfer amounts to 0.96%/h in wild type cells and to 0.54%/h in the proteinase yscA-proteinase yscB double mutant. Thus, 34% (first hour) and 44% (after the first hour) of degenerative capacity is lacking in the double mutant and must thus be due to the two vacuolar enzymes.

Furthermore, the question was addressed whether the two vacuolar endopeptidases are involved in the degradation of falsely synthesized proteins. The degradation of false proteins is essential for the cell to avoid accumulation of protein waste and their degradation proceeds rather rapidly (45, 46). The arginine analogue canavanine was used to induce the synthe-sis of abnormal proteins in yeast (47). Cells were grown on MV-medium into logarithmic phase and treated for 0.5 h with canavanine prior to labeling with [3H]leucine (0.5 h) as described above. Degradation of protein in wild type cells proceeded in a nearly linear manner of about 2%/h (Fig. 2B). Within the time period of the first 0.5 h, no difference in the degradation rate was found for wild type and the double

![Fig. 1. Protein degradation in wild type and proteinase-deficient mutant cells under starvation (sporulation) conditions.](image)

Mutants with the genotypic designation pep4-3 had been isolated (24) which lack proteinase yscA and are deficient in the proteolytic maturation of proteinase yscB (27) and car-boxypeptidase yscY (42). Recently, it has been shown that the PEP4 gene codes for proteinase yscA (8, 9) and is thus allelic to the PRA1 gene (10). For pep4-3 strains, a turnover value of 30-38% as compared with wild type was observed under sporulation conditions (43). Proteinase yscA, proteinase yscB, and carboxypeptidase yscY have been shown to be synthesized as precursor molecules (10, 11, 28, 42). Proteinase yscA turned out to be the trigger of the maturation event of the 42-kDa precursor of proteinase yscB and of the precursor of carboxypeptidase yscY to yield the active enzymes (8-11). The difference in the phenotypic effects of the pral-1 and pep4-3 mutant alleles of proteinase yscA resides in the ability of the pral-1 mutant to partly process the 42-kDa precursor of proteinase yscB to the mature enzyme which subsequently processes carboxypeptidase yscY, whereas the pep4-3 mutant is unable to do so (10). Astounding, even though the pral-1 mutant carries some mature proteinase yscB while the pep4-3 mutant does not, there is no significant difference in protein degradation rates in the pral-1 mutant (Fig. 1) and in the pep4-3 mutant (43). In contrast, the double mutant pral-1 prbl-1 carrying deficiencies in both proteinase activities, yscA and yscB, shows with a rate of 15% of wild type a considerably lower degradation rate than the pep4-3 mutant. This phenomenon might be explained by the fact that even though the pep4-3 mutant does not carry mature proteinase yscB, it contains an intact 42-kDa precursor of proteinase yscB, which has recently been shown to be capable of degrading protein (11), whereas the pral-1 prbl-1 double mutant harbors muta-ted, inactive proteinase yscB. However, it cannot be com-3pletely excluded that the different degradation rates in pep4-3 mutant cells and in pral-1 prbl-1 double mutants is only due to the different genetic background of both strains.

We furthermore investigated whether the drastic decrease of the degradation capacity of 85% in pral-1 prbl-1 double mutants was only due to the absence of proteinase yscA and proteinase yscB activities and not in part due to the additional absence of carboxypeptidase yscY which is contained in this mutant as inactive precursor. For this reason we measured protein degradation in a proteinase yscA-proteinase yscB double mutant (pral-2 prbl-1) carrying another mutated proteinase yscA allele pral-2, which also in the absence of proteinase yscB is able to at least partly activate carboxypeptidase yscY (not shown). A residual protein degradation rate of 10% of wild type was found, indicating that carboxypeptidase yscY was not responsible for the drop in protein degrad-3ving capacity in pral-1 prbl-1 mutants. Carboxypeptidase yscY acts most likely on the peptides which are generated by proteinase yscA and proteinase yscB.

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Lysosomal Proteinases Are Essential in the Yeast Life Cycle

Sporelation frequency of proteinase defective mutants of *S. cerevisiae* on different media

Diploid cells were subjected to sporulation conditions as described under “Materials and Methods.” Data are given as average values from several experiments using different strains of the same genotypes. All diploid strains are described in Table I. AA, amino acids.

| Genotype                  | Missing activity     | Medium       | Sporulation frequency (% of wild type) |
|---------------------------|----------------------|--------------|---------------------------------------|
| *MATa PRA1 PRB1*          | None                 | SP           | 100 (control)                         |
| *MATa pra1-1 PRB1*        | Proteinase yscA      | SP           | 21                                    |
| *MATa pra1-1 PRB1*        | Proteinase yscB      | SP           | 26                                    |
| *MATa pra1-1 prb1-1*      | Proteinase yscA      | SP           | <0.1                                  |
| *MATa pra1-1 prb1-1*      | Proteinase yscB      | SP           | <0.1                                  |
| *MATa pra1-1 prb1-1*      | Carboxypeptidase yscY* | SP           | 100 (control)                         |
| *MATa PRA1 PRB1 gua1*     | None                 | SP, SP + AA, MNA, YPA | <0.1                                  |
| *MATa pra1-1 prb1-1 gua1* | Proteinase yscA      | SP, SP + AA, MNA, YPA | <0.1                                  |
| *MATa pra1-1 prb1-1 gua1* | Proteinase yscB      | SP, SP + AA, MNA, YPA | <0.1                                  |
| *MATa pra1-2 prb1-1 gua1* | Proteinase yscA      | SP           | <0.1                                  |
| *MATa pra1-2 prb1-1 gua1* | Proteinase yscB      | SP           | <0.1                                  |

* Carboxypeptidase yscY only present as inactive precursor (10).

Consequences of Proteinase yscA and Proteinase yscB Deficiency on Cell Physiology—In the absence of proteinase yscA and proteinase yscB, differences in protein degradation were greatest under conditions of nutritional stress. Therefore, we expected these two endopeptidases to have the most pronounced effects on cell physiology under these conditions. This was indeed the case. No differences in growth rate between wild type and the proteinase yscA-proteinase yscB double mutant (pra1-1 prb1-1) were visible during the vegetative growth phase (net shown). However, the differentiation process of sporulation was dramatically affected (Table II). Whereas, in comparison to wild type, the sporulation frequency of diploids homozygously deficient in proteinase yscA (allele pra1-1) was reduced between 40% (21) and 79% (this study) and of diploids homozygously deficient in proteinase yscB (allele prb1-1) was reduced between 50% (20) and 74% (this study), diploids lacking both endopeptidases, proteinase yscA and proteinase yscB, completely ceased to sporulate (Table II). Thus, the presence of proteinase yscA and proteinase yscB is necessary for sporulation. Sporulation of diploid cells requires a nitrogen-free medium. New protein synthesis is required for the formation of spores and is dependent on the intracellular amino acid pool and, when depleted, on the supply of amino acids by protein degradation at the cost of unneeded vegetative protein. Lack of proteinases yscA and yscB leads to a severe impairment of protein degradation (Fig. 1) which might lead to a block in amino acid supply, preventing the synthesis of spore proteins. This hypothesis was tested by an external supply of amino acids to cells transferred onto sporulation medium. No spore formation was visible in the proteinase yscA-proteinase yscB double mutant and, as found previously by others (43), the sporulation frequency of wild type cells decreased dramatically. Recently, the drop of the intracellular GTP pool was reported to trigger initiation of sporulation (30, 48). Guanine auxotrophic strains sporulate on guanine-depleted acetate medium containing nitrogen or amino acids (30). Thus, if the lack of protein degradation and supply of amino acids were the only reason for the defective sporulation event in proteinase yscA-proteinase yscB (pra1-1 prb1-1) double mutants, supply of amino acids should restore sporulation of the proteinase yscA-proteinase yscB double mutants carrying the guanine auxotrophy marker. However, in contrast to guanine auxotrophs wild type for proteinases yscA and yscB, no formation of asci was found in homozygous pra1-1 prb1-1 gua1-1 diploids in guanine-depleted, amino acid-containing sporulation medium (Table II). This behavior of the mutants cannot be due to a defective uptake of amino acids as radioactively labeled leucine was taken up in the mutants with a comparable rate as in wild type cells (not shown). Thus, impaired protein degradation and subsequent lack of amino acid supply can at least not be the only reason for the defect in sporulation of proteinase yscA-proteinase yscB double mutant cells.

*pra1-1 prb1-1* double mutants have been shown to be defective in maturation and activation of carboxypeptidase yscY (10) and possibly of other soluble vacuolar hydrolases.2 Proteinase yscA mutants carrying the allele pep4-3, which are unable to mature proteinase yscB and carboxypeptidase yscY (27, 42), were also found to be defective in sporulation (43). Possibly, it is the lack of maturation of one or more of the vacuolar enzymes needed for the sporulation process to occur that prevents the proteinase-deficient strains from sporulating. This missing enzyme activity cannot solely be due to carboxypeptidase yscY because guanine auxotrophic proteinase yscA-proteinase yscB double mutants carrying the *pra1-2* allele instead of the *pra1-1* allele, and due to this reason have active carboxypeptidase yscY,3 are also unable to sporulate on guanine-depleted amino acids containing sporulation medium (Table II).

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1 H. H. Hirsch, unpublished observations.
2 B. Mechler, unpublished observations.
Another reason for the sporulation deficiency of proteinase yscA-proteinase yscB double mutants might reside in their inability to inactivate vegetative protein(s) which are harmful for the sporulation process.

Nitrogen starvation induces a nutritional stress on cells and leads to a rearrangement of the protein and RNA content in their viability nearly completely within 3-4 days (Fig. 3). Proteinase yscB mutants behaved like wild type. No loss in viability was visible during nitrogen starvation for more than 8 days without loss of viability, strains deficient in proteinase yscA lost their viability nearly completely within 3-4 days (Fig. 3). Double mutants carrying the proteinase yscA mutation and, in addition, the proteinase yscB mutation, behaved similarly to the mutant deficient in proteinase yscA alone. Viability was reproducibly lost within 3-4 days (Fig. 3). Also starvation for carbon, sulfur, and phosphate resulted in a dramatically increased loss of viability of proteinase yscA mutant strains as compared with wild type (not shown). Thus, proteinase yscA is a crucial enzyme, vital for cell survival under nutritional stress conditions.

Vacuolar Proteinase Deficiency and Degradation of Distinct Proteins—Even though degradation of proteins is a well-established process, the degradation of only a very few distinct proteins has been characterized up to now in yeast. Among those are the glucose-regulated enzyme fructose-1,6-biphosphatase which undergoes inactivation upon transfer of cells from a nonfermentable carbon source to glucose (50-52), and two mutated proteins, a mutated carboxypeptidase yscY (allele prcl-1) (18, 27) and a mutated fatty acid synthetase (53). The obvious question to ask is: are the vacuolar endopeptidases proteinase yscA and proteinase yscB involved in these proteolytic processes? Previous studies had shown that fructose-1,6-biphosphatase undergoes catabolite inactivation in two steps. A rapid phosphorylation step is followed by disappearance of fructose-1,6-biphosphatase (51, 54). Even though it has been demonstrated previously that the vacuolar proteinases yscA and yscB were not involved in the in vivo inactivation event at the level of enzyme activity (1, 2, 5, 6) by analyzing the effect of single proteinase mutations, no data are available on the combined effect of both proteinases on the degradation of the fructose-1,6-biphosphatase in vivo. Measuring fructose-1,6-biphosphatase activity, Funagama et al. (55) reported recently that in mutant cells carrying the pep4-3 mutation, and thus being devoid of proteinase yscA and in turn blocked in the maturation of other vacuolar soluble peptidases (8-11), the proteolytic inactivation step of fructose-1,6-biphosphatase is absent. We followed the inactivation of fructose-1,6-biphosphatase in double mutant cells deficient in both vacuolar peptidase activities, proteinase yscA (allele prcl-1) and proteinase yscB. Both mutations together lead to an even tighter phenotype concerning the lack of maturation of other vacuolar hydrolases as does the proteinase yscA mutant carrying the pep4-3 allele.4 Inactivation of fructose-1,6-biphosphatase was determined on the basis of enzyme activity (Fig. 4). As can be seen, fructose-1,6-biphosphatase activity disappears in a similar fashion in the proteinase yscA-proteinase yscB double mutant as in wild type cells (Fig. 4). When following the fate of the fructose-1,6-biphosphatase by immunoblotting in the same crude extracts used for enzyme tests, the protein disappeared in a similar manner in wild type and the proteinase double mutant (not shown). Radioactive labeling of cell protein and precipitation of fructose-1,6-biphosphatase with specific antibodies shows an equivalent pattern when a mutant carrying a disrupted proteinase yscA gene (prcl-1:URA3) is compared with its isogenic wild type counterpart. In both cell types fructose-1,6-biphosphatase disappears with the time of inactivation (Fig. 5). In both experiments (Figs. 4 and 5) inactivation and

4 H. H. Hirsch and D. H. Wolf, unpublished observations.
degradation of fructose-1,6-bisphosphatase seems to proceed slightly slower in the mutant cells as compared with the wild type controls. Whether this points to some common role of the vacuolar endopeptidases in fructose-1,6-bisphosphatase degradation or whether this is due to strain differences cannot be answered. Clearly, however, the two vacuolar endopeptidases yscA and yscB are not the only enzymes responsible for catabolite inactivation of fructose-1,6-bisphosphatase.

Schweizer et al. (53, 56) proposed that fatty acid synthetase assembly from its two subunits α and β is sensitive to localized conformational changes. Mutational alterations destabilize the fatty acid synthetase complex and lead to degradation of the subunits (53, 57). A missense mutation (fasl-248) in the β-subunit of fatty acid synthetase leads to wild type amounts of mRNA for the β-subunit but no protein is detectable (57). In addition, the unassembled α-subunit disappears in the mutant of the β-subunit (allele fasl-248) (57). We checked for involvement of the two vacuolar endopeptidases proteinase yscA and proteinase yscB in the possible degradation of the mutated β- and the nonassembled α-subunit. As can be seen in Fig. 6, the mutated β-subunit of the fatty acid synthetase complex is not visible at all (lower arrow), and the nonassembled α-subunit of the enzyme disappears in stationary phase in the strain carrying the fasl-248 mutation together with deficiencies in both vacuolar endopeptidases (upper arrow). As the two vacuolar carboxypeptidases yscY and yscS are missing in addition in the mutant strain used (UT-PDF-7A), these two carboxypeptidases cannot be involved in the degradation process of the fatty acid synthetase subunits.

Carboxypeptidase yscY is synthesized as an inactive precursor molecule which travels via part of the secretory pathway into the vacuole (42, 58). We had isolated a structural gene mutation (prcl-1) in the vacuolar carboxypeptidase yscY (18, 59), which resulted in a precursor protein of the enzyme that is not processed to its mature form (27). This protein is rather quickly degraded in cells wild type for other peptidases (27). This carboxypeptidase yscY mutant protein was analyzed in more detail. As can be seen in Fig. 7, mutant prcl-1 carboxypeptidase yscY appears as a precursor molecule of M = 67,000 on SDS-PAGE (Fig. 7, compare lane 1 (prcl-1 mutant), lower arrow, with lane 3 (wild type asterisk)). In contrast, wild type carboxypeptidase yscY appears as a 69-kDa precursor form (Fig. 7, lanes 2 and 3, upper arrow). In cells wild type for other peptidases, this 69-kDa precursor of carboxypeptidase yscY is proteolytically transferred into the 61-kDa mature protein (Fig. 7, lane 3, asterisk). Mutants deficient in proteinase yscA block this maturation process and accumulate 69-kDa wild type carboxypeptidase yscY precursor (Fig. 7, lane 2, upper arrow). The 67-kDa prcl-1 mutant carboxypeptidase yscY precursor is never matured (Fig. 7, lane 1). The two bands of lower molecular weight in lanes 1 and 2 are not due to correctly processed carboxypeptidase yscY. They are most likely due to degradation products of the carboxypeptidase yscY protein.

Carboxypeptidase yscY carries four N-linked carbohydrate chains (60, 61). The question had to be answered whether the difference in molecular weight of 2 kDa of the prcl-1 mutant precursor of carboxypeptidase yscY and the wild type precursor form was due to a difference in glycosylation of the proteins or due to a difference in the molecular weight of the peptide chains. Tunicamycin treatment of cells prevents N-linked carbohydrate chains (60, 61). The question had to be answered whether the difference in molecular weight of 2 kDa of the prcl-1 mutant precursor of carboxypeptidase yscY and the wild type precursor form was due to a difference in glycosylation of the proteins or due to a difference in the molecular weight of the peptide chains. Tunicamycin treatment of cells prevents N-linked carbohydrate chains (60, 61).
glycosylation of proteins passing through the organelles of the secretory pathway (62). As can be seen in Fig. 8, tunicamycin treatment of the carboxypeptidase yscY (prcl-1) mutant cells and of proteinase yscA mutant cells accumulating the wild type precursor of carboxypeptidase yscY leads to procarboxypeptidase yscY proteins of similar molecular weight of M. = 61,000. This indicates a different glycosylation pattern of the prcl-1 mutant and wild type precursor forms. Thus, the prcl-1 mutation does not lead to gross changes of the size of the procarboxypeptidase yscY protein. The glycosylated precursor of the prcl-1 mutant (Fig. 7, lane 1, lower arrow) seems to have the same molecular weight as the wild type carboxypeptidase yscY precursor found in sec18 mutant cells (Fig. 7, lane 4) which are deficient for the delivery of secretory proteins from the endoplasmic reticulum to the Golgi apparatus (58, 63) at elevated temperature (37 °C). In these cells, procarboxypeptidase yscY is only core glycosylated and the Golgi-specific carbohydrate modifications are missing (58). It thus appears that the prcl-1 mutation leads to the "ER-form" of the carboxypeptidase yscY precursor (Fig. 7). As wild type and prcl-1 mutant procarboxypeptidase yscY proteins accumulate as precursors of similar molecular weight in sec18 mutant cells at restrictive temperature (not shown), it seems evident that both proteins are similarly core glycosylated. Thus, the difference in molecular mass of 2 kDa of the prcl-1 mutant precursor and the wild type carboxypeptidase yscY precursor in a secretion wild type background seems to be due to differences in the carbohydrate trimming reactions occurring in the Golgi apparatus. At least two possibilities have to be taken into account: 1) the prcl-1 mutant carboxypeptidase yscY is resistant to trimming in the Golgi apparatus or 2) the prcl-1 mutant precursor never reaches the Golgi apparatus.

We measured the half-life of mutated prcl-1 carboxypeptidase yscY precursor protein in cells otherwise wild type for peptidases and found it to be approximately 30 min (Fig. 9). We checked whether the vacuolar endopeptidases proteinase yscA and proteinase yscB were involved in this degradation event of the mutated carboxypeptidase yscY protein by crossing these mutations into the prcl-1 mutant strain. As can be seen in Fig. 9, the half-life of 30 min of the mutated protein is not altered by the absence of the two vacuolar endopeptidases, indicating that they are not involved in the degradation process. As the mutant also carried the carboxypeptidase yscS mutation, this enzyme can also not be involved in the degradation of the false carboxypeptidase yscY protein.

As stated above, the false carboxypeptidase yscY protein enters the secretory pathway. Mutated carboxypeptidase yscY accumulates in sec18 mutant cells defective in the delivery of secretory proteins from the endoplasmic reticulum to the Golgi apparatus under restrictive conditions (Fig. 10A). In contrast to cells wild type for the secretory defect (Fig. 10B), the false carboxypeptidase yscY precursor does not disappear in sec18 cells under these conditions (Fig. 10A). There might be at least two explanations for this phenomenon: 1) the false carboxypeptidase yscY precursor is degraded in the endoplasmic reticulum but at the restrictive temperature for the sec18 mutation, the peptidase responsible for degradation is inactivated. 2) Under restrictive conditions the sec18 mutation blocks further delivery of the false procarboxypeptidase yscY protein to the compartment where it is normally degraded.

We consider the first possibility, inactivation of the degradative peptidase at restrictive temperature of the sec18 mutation, unlikely, and we favor the second possibility. Here, upon release from the restrictive conditions the false procarboxypeptidase yscY protein may travel through further compartments of the secretory pathway to the compartment of degradation but fails to be trimmed at its carbohydrate chains in the Golgi apparatus. Alternatively, the false carboxypeptidase yscY precursor never reaches the Golgi apparatus and is degraded in a pre-Golgi compartment to which protein delivery is still under the control of the sec18 protein.

**DISCUSSION**

This study shows that the two vacuolar endopeptidases, proteinase yscA and proteinase yscB, constitute the major proteolytic catalysts in the yeast cell under nutritional stress conditions, which trigger degradation of proteins down to trihydroxacetic acid-soluble products. Both endopeptidases contribute nearly equally to the degradation event under the...
nutritional stress of poor carbon supply (acetate) and nitrogen deprivation, constituting between 65 and 70%, respectively, of the degradative capacity in vivo. The degradative capacity of proteinase yscA and proteinase yscB overlaps, as the absence of both endopeptidases leads to the lack of 86% of the degrading capacity under these conditions. The degrading capacity of both endopeptidases in vivo is reflected by their in vitro activity. Both enzymes together are responsible for between 86 and 97% of hydrolysis of yeast protein and methyalcohol, respectively, in crude extracts of stationary phase cells. In vivo about 14% of protein degradation under the nutritional stress applied are not due to the action of proteinase yscA and proteinase yscB and must thus be due to other proteolytic systems.

When cells grow vegetatively on glucose, protein degradation is high during the first hour and decreases after this time period to about half of that of stationary phase cells. As expected from the much lower amounts of the two vacuolar endopeptidases when cells grow vegetatively on glucose, their participation in protein degradation is considerably less than 50% under these conditions.

As previously shown (47), induction of false proteins leads to considerably increased protein degradation. As shown here, after the time period of 1 h, the degradation rate is about twice as high when canavanine is added to vegetatively growing wild type cells (Fig. 2, A and B). Under these conditions, proteinase yscA and proteinase yscB have also some impact on the degradation process. However, more than 60% of the degradative capacity of the cells must be due to different proteolytic systems under these conditions (Fig. 2B). One such system, the ubiquitin-dependent proteolytic system has clearly been identified in yeast recently (38). In addition, many more proteases besides the known vacuolar ones have been found (64-66, for reviews, see Refs. 1 and 2).

The presence of proteolytic systems responsible for protein degradation which are different from the vacuolar endopeptidases proteinase yscA and proteinase yscB is also obvious from the study of the degradation behavior of mutated proteins of fatty acid synthetase (Fig. 6) and carboxypeptidase yscY (Fig. 9) as well as the catalytic degradation of the gluconogenic enzyme fructose-1,6-bisphosphate (Figs. 4 and 5). These proteins are also degraded in the absence of the two vacuolar endopeptidases. The previous claim of Funagama et al. (55) that fructose-1,6-bisphosphatase does not undergo proteolytic degradation in a pep4-3 mutant strain deficient in proteinase yscA could not be substantiated under the conditions used in our experiments. There is a slight reduction in the catalytic degradation of fructose-1,6-bisphosphatase visible in mutants completely deficient in proteinase yscA due to disruption of its gene or strains carrying mutations in proteinase yscA and proteinase yscB (Figs. 4 and 5). However, as fructose-1,6-bisphosphatase does undergo degradation in these mutants, proteinase yscA and proteinase yscB do not seem to be the crucial enzymes triggering catalebolite degradation of fructose-1,6-bisphosphatase (Figs. 4 and 5). The importance of the growth conditions of the proteinase yscA-deficient pep4-3 mutant cells prior to glucose addition to achieve inactivation of fructose-1,6-bisphosphatase has been pointed out recently (67).

The absence of vacuolar peptidases has a dramatic effect on yeast physiology. Mutants deficient in the endopeptidases proteinase yscA and proteinase yscB show a considerably reduced sporulation activity (Table II) (20, 21). Deficiency in both endopeptidases leads to a complete loss of diploid cells to sporulate (Table I). We had previously shown that the two vacuolar carboxypeptidases yscY and yscS are also enzymes necessary for sporulation. However, only when the vacuolar endopeptidase, proteinase yscB, and the carboxypeptidases are absent can this requirement be observed (22).

What is the reason for the sporulation negative phenotype of the protease-deficient mutants? One explanation for this phenotype is the lack of provision of amino acids for protein synthesis needed for the generation of the four spores at the expense of unneeded vegetative protein. Supply of amino acids seems to be an attractive explanation for the function of proteinase yscB and the two carboxypeptidases yscY and yscS in vivo. As found in vivo, proteinase yscB might degrade proteins in vitro into peptides, which are subsequently hydrolyzed by carboxypeptidase yscY and carboxypeptidase yscS into the respective amino acids. The occurrence of this sequence of events might be indicated by the cumulative effect in dramatically decreasing the rate of sporulation of cells when the proteinase yscB mutation, which itself does not lead to cessation of sporulation, is introduced into a strain harboring the two carboxypeptidases yscY and yscS mutations, which themselves do not affect sporulation (22).

However, also the accumulation of vegetative proteins or peptides unfavorable for sporulation might be the reason for the disturbed sporulation of proteinase yscB and carboxypeptidases yscY- and yscS-deficient mutants. The function of proteinase yscA in the sporulation event is even less clear. As this enzyme constitutes the initiating trigger protein for processing and activation of other soluble vacuolar hydrolases (8-11), absence of proteinase yscA might disturb different functions. (i) Vacuolar hydrolases necessary for sporulation might not be processed and activated and thus be unable to serve their functions; (ii) absence of proteinase yscA might disturb protein degradation directly and by this block amino acid supply for new spore protein synthesis; (iii) lack of proteinase yscA might lead to an accumulation of vegetative proteins incompatible with sporulation; (iv) all the above mentioned possibilities might apply. The fact that guanine depletion, which initiates sporulation in wild type cells under amino acid and nitrogen supply (30, 48) does not restore sporulation in proteinase yscA-proteinase yscB mutant cells under the same conditions, indicates that some disturbed event other than only amino acid supply is responsible for sporulation deficiency in mutants devoid of these two enzymes.

The very special role that proteinase yscA plays in cell physiology is demonstrated by the fact that lack of this enzyme leads to cell death under conditions of nutrient deprivation (Fig. 3). The central function triggered by proteinase yscA cannot be substituted by proteinase yscB. Lack of this enzyme does not affect cell survival. The reason for the central role of proteinase yscA under starvation conditions is at present unknown. One might suspect that it is not the diminished protein degradation rate per se that is responsible for cell death in proteinase yscA-deficient mutants as mutations devoid of proteinase yscB, showing a similarly diminished protein degradation rate as do proteinase yscA mutants, do not die during the time period analyzed. One reason for the central role of proteinase yscA in starving cells might reside in its central function in the initiation of processing of vacuolar enzymes to their mature species (8-11), an event defective in proteinase yscA mutants. The active vacuolar enzymes might be necessary for survival. Another reason for the detrimental effect of proteinase yscA absence in mutant cells might be the harmful accumulation of proteins which are targets of proteinase yscA destruction in wild type cells. The elucidation of the diverse functional roles of proteinase yscA in cell physiology rests on future studies.

Acknowledgments—We thank Dr. Ernst Freese (Bethesda, MD),
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Lysosomal Proteinases Are Essential in the Yeast Life Cycle

Table 1

Saccharomyces cerevisiae strains used

| Strains           | Source or reference                  |
|-------------------|--------------------------------------|
| YX110-1A          | Yeast Genetic Stock Center           |
| 2860              |                                      |
| 200-12            |                                      |
| W303-13           |                                      |
| K68               |                                      |
| YA4               |                                      |
| FA2-10            |                                      |
| FA4               |                                      |
| FA4-10            |                                      |
| FA19              |                                      |
| FA19-10           |                                      |
| FA2-10            |                                      |
| FA2-10            |                                      |
| FA4               |                                      |
| FA4               |                                      |
| FA4-10            |                                      |
| FA19              |                                      |
| FA19-10           |                                      |
| FA2-10            |                                      |
| FA2-10            |                                      |
| FA4               |                                      |
| FA4               |                                      |
| FA4-10            |                                      |
| FA19              |                                      |
| FA19-10           |                                      |
| FA2-10            |                                      |
| FA2-10            |                                      |

Preparation of cell extracts: Preparation of cell extracts was done as described [31]. Before using 0.3 M potassium phosphate buffer, pH 7.1 for resuspending cells, when fractionation buffers were employed in fractionation buffers were employed in in vivo experiments of yeast lysosomes. The cell-free extracts were obtained by addition of 0.1 M sucrose, 0.01 M EDTA, pH 7.1. Trichloroacetic acid was added to the extracts to a final concentration of 5%. Proteinase activity was assayed according to Schekman and Pieler [32] in extracts activated by addition of 2.5% SDS (2 M NaCl) using acrylamide as a substrate.

Carnitine-phosphate was assayed according to Abara et al. [33] using 0.01 M tricarboxylic acid as substrate.

Protein determination: Protein determination was measured by the method of Lowry et al. [34] using bovine serum albumin as standard.

In vivo degradation of fatty acid synthetase: For in vivo degradation of fatty acid synthetase, cells grown on agar medium were collected by centrifugation (3 min 3000 g) at 4 °C and resuspended in 0.1 M potassium phosphate buffer. Samples were routinely mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate. The resulting suspension was mixed with 0.2 ml of 0.5 M sodium carbonate and 0.2 ml of 0.5 M sodium bicarbonate.