Selective Inhibitors of the Proteasome-dependent and Vacular Pathways of Protein Degradation in Saccharomyces cerevisiae*

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We have studied whether various agents that inhibit purified yeast and mammalian 26 S proteasome can suppress the breakdown of different classes of proteins in Saccharomyces cerevisiae. The degradation of short-lived proteins was inhibited reversibly by peptide aldehyde inhibitors of proteasomes, carboxoyl-leucinyl-leucinyl-leucinyl-leucinyl-leucinyl-leucinyl-leucinyl-norvalinal (MG132) and carboxoyl-leucinyl-leucinyl-leucinyl-norvalinal (MG115), in a yeast mutant with enhanced permeability, but not in wild-type strains. Lactacystin, an irreversible proteasome inhibitor, had no effect, but the β-lactone derivative of lactacystin, which directly reacts with proteasomes, inhibited the degradation of short-lived proteins. These inhibitors also blocked the rapid ubiquitin-dependent breakdown of a β-galactosidase fusion protein and caused accumulation of enzymatically active molecules in cells. The degradation of the bulk of cell proteins, which are long-lived molecules, was not blocked by proteasome inhibitors, but could be blocked by phenylmethylsulfonyl fluoride. This agent, which inhibits multiple vacuolar proteases, did not affect the proteasome or breakdown of short-lived proteins. These two classes of inhibitors can thus be used to distinguish the cytosolic and vacuolar proteolytic pathways and to increase the cellular content of short-lived proteins.

Eukaryotic cells contain two distinct systems for protein degradation: the vacuolar system in plants and yeast, which corresponds to the lysosomes in mammals, and the soluble ATP-requiring system that involves ubiquitin and the proteasome (1–4). In the ubiquitin-proteasome pathway, substrates are first marked for degradation by conjugation to multiple molecules of ubiquitin and then are hydrolyzed by the 26 S proteasome complex (2, 5). One of the critical functions of this pathway is the rapid degradation of highly abnormal proteins as may arise by mutations, postsynthetic damage, or genetic processes, including antigen presentation on major histocompatibility class I molecules (8), the processing of NF-κB (10), and the turnover of membrane proteins (11). A distinct type of proteasome inhibitor is lactacystin, a Streptomyces metabolite, isolated by Omura et al. (12). This agent selectively inhibits multiple peptidase activities of mammalian proteasomes by covalently modifying the active site threonine residues of the β-subunits (13). The active chemical species which reacts with the proteasome is not lactacystin, but its spontaneous hydrolysis product clasto-lactacystin β-lactone (hereafter called β-lactone) (14). In many different mammalian cells, treatment with lactacystin inhibits overall protein degradation, including the breakdown of short-lived and long-lived components (11, 15, 16).

The budding yeast Saccharomyces cerevisiae has been extensively used for genetic and physiological analysis of the ubiquitin-proteasome pathway (3, 7, 17). Because of the many important functions of the proteasome and of ubiquitination independent of the proteasome, mutants affecting ubiquitin conjugation and the proteasome can have pleiotropic physiological effects and lead to secondary compensations or suppressor mutations. Therefore agents that might rapidly and reversibly inhibit proteolysis in yeast by blocking proteasome activity would be very useful for many studies. In addition, such inhibitors might be useful to promote the accumulation of short-lived proteins in cells. The present study demonstrates that inhibitors of proteasome function can be used to reduce protein breakdown in growing yeast and that these agents and an inhibitor of yeast vacuolar proteases, most of which are serine proteases, can be used to clarify the roles of these two distinct proteolytic systems in degradation of different cell proteins.

EXPERIMENTAL PROCEDURES

Saccharomyces cerevisiae Strains and Plasmid—S. cerevisiae strains used in this study were JN284 (MATa, his7, leu2, ura3, isel, kindly provided by Dr. J. C. Wang, Harvard University) and W303 (MATa, ade2, his3, leu2, trp1, ura3, can1, laboratory stock). The plasmid carrying ubiquitin-proline-β-galactosidase was a gift from Dr. S. Jentsch (University of Heidelberg).

Proteasome Inhibitors—N-acetyl-leucinyl-leucinyl-norleucinal (MG101, also known as calpain inhibitor-1),¹ carboxoyl-leucinyl-leucinyl-leucinyl-norvalinal (MG115), carboxoyl-leucinyl-leucinyl-2-naph-thol-alanal, carboxoyl-leucinyl-leucinyl-norleucinal, carboxoyl-xyl-leucinyl-leucinyl-leucinyl (MG132), and clasto-lactacystin β-lactone were provided by Proscript (formerly Myogenics, Cambridge, MA). Lactacystin was kindly provided by Dr. S. Omura (The Kitasato Institute, Heidelberg). The abbreviations used are: MG101 (Nac-Lle-Lal), N-acetyl-leucinyl-leucinyl-norleucinal (also known as calpain inhibitor-1); MG115 (Cbz-Lle-Lal), carboxoyl-leucinyl-leucinyl-norvalinal; MG132 (Cbz-LLal), carboxoyl-leucinyl-leucinyl-leucinyl; Ub-P-β-gal, ubiquitin-proline-β-galactosidase; Suc-LVY-AMC, succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin; PMSF, phenylmethylsulfonyl fluoride.

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Tokyo). These inhibitors (dissolved in Me$_2$SO) were added to yeast cells 90 min prior to labeling and also included in the medium during the chase period. Final concentrations of Me$_2$SO were 0.1–0.2% in treated and control cells.

**Measurement of Protein Degradation**—The breakdown of short-lived and long-lived proteins in exponentially growing yeast cells was measured as described by Lee et al. (18) except that 0.5 μCi of $[^{14}$C]leucine (Amersham Corp.) was used as a radioactive amino acid. For determination of the half-life of ubiquitin-proline-$\beta$-galactosidase (Ub-$\beta$-gal) in yeast cells, pulse labeling and immunoprecipitation with anti-$\beta$-gal antibody were performed as described by Bachmair et al. (19).

**Other Methods**—All manipulation of yeast cell and measurement of $\beta$-galactosidase activity in yeast cell using o-nitrophenyl-$\beta$-n-galactopyranoside as the substrate were carried out by Ausubel et al. (20).

Hydrolysis of peptide substrate (Suc-LLVY-AMC) and degradation of ubiquitin-$^{125}$I-lysozyme by purified yeast 26 S proteasomes were measured as described by Rock et al. (8). Protein concentrations were determined by the Bradford method (Pierce).

**RESULTS**

**Effect of Peptide Aldehydes on Degradation of Short-lived Proteins**—The proteasomes purified from wild-type *S. cerevisiae*, like their counterparts from mammalian cells, are sensitive to proteasome inhibitors, such as peptide aldehydes. At 10 μM, peptide aldehydes blocked almost completely the hydrolysis of the fluorescein peptide substrate, Suc-LLVY-AMC, and markedly reduced degradation of ubiquitin-conjugated $^{125}$I-lysozyme by purified yeast 26 S proteasomes (Table I). As found previously (10), Cbz-LLLal (MG132) was more potent than Cbz-LLnVal (MG115) and lactacystin (Table I). However, our initial attempts to demonstrate an effect of these peptide aldehyde inhibitors were unsuccessful in wild-type yeast cells, apparently because of the impermeability of these cells to the inhibitors. *S. cerevisiae* are resistant to many drugs and metabolic inhibitors because of a failure of these agents to penetrate the outer wall or cell membranes (21, 22). Several mutant strains of yeast with enhanced permeability to drugs have been described. For example, one of these strains, isel (erg6) strain, is permeable to antitumor drugs, such as topoisomerase inhibitors, and to protein synthesis inhibitors (21, 23). Therefore we tested whether proteolysis in this mutant strain is affected by the inhibitors of proteasome.

In the isel mutant strain, the degradation of pulse-labeled proteins was reduced by 50–60% with 50 μM of MG132, which did not affect the degradation of these proteins in wild-type cells (W303) (Fig. 1A). In the absence of MG132, this mutant cell showed similar rates of protein degradation as wild-type cell (Fig. 1A). Although the 10-min pulse preferentially labels short-lived proteins, it also necessarily labels many long-lived proteins, which usually comprise about 20% of the proteins labeled under these conditions (18). If the fraction of these long-lived proteins (whose degradation was not affected by MG132, see below) is subtracted from the total labeled pro-
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**Table II**

Effect of various peptide aldehydes on the degradation of pulse-labeled proteins in ise1 cells

| Peptide aldehyde (50 μM) | Inhibition (%) |
|--------------------------|----------------|
| None                     | 0              |
| Cbz-Leu-Leu-leucinal (MG132) | 59            |
| Cbz-Leu-Leu-norvalinal (MG115) | 41            |
| NAc-Leu-Leu-norleucinal (MG101) | 0            |
| Cbz-Leu-Leu-naphthol-alanal | 0             |
| Cbz-Leu-Leu-α-norleucinal | 0             |

Fig. 2. Effect of lactacystin and β-lactone on the degradation of pulse-labeled proteins in ise1 cells. 20 μM lactacystin or 20 μM β-lactone was added to ise1 cells for 90 min prior to labeling, and their effect on the degradation of 10-min pulse-labeled proteins was measured during the chase period.

Fig. 3. Effect of proteasome inhibitors on the rapid breakdown of the fusion polypeptide Ub-P-β-gal. A, effects of various proteasome inhibitors (50 μM MG132, 20 μM lactacystin, or 20 μM β-lactone) on the breakdown of Ub-P-β-gal. For control, 0.1–0.2% Me_SO was added to cells. B, MG132 (50 μM) caused accumulation of Ub-P-β-gal in enzymatically active form in the ise1 cells. After 2 h of incubation, cells were resuspended in SD medium with or without MG132 and incubated for an additional 2 h. The β-galactosidase activity was measured as described by Ausubel et al. (20).

Since the proteasome inhibitors can block the rapid breakdown of proteins by the ubiquitin pathway without affecting protein synthesis, they should cause short-lived proteins to accumulate, perhaps in active form. To test this possibility, we used the ise1 cells transfected with Ub-P-β-gal and assayed β-galactosidase activity in the cells (an inhibition of degradation should lead to higher protein level and greater enzyme activity). After addition of MG132, this activity increased steadily and did not change in untreated cells (Fig. 3B). After 2 h, treated cells were collected, washed, and were resuspended with or without inhibitor. With MG132 present, the β-galactosidase activity continued to increase in the cells, but slowly decreased after the inhibitor was removed (Fig. 3B). Thus these agents can be used to increase the content of proteins that are normally degraded rapidly by the ubiquitin-proteasome pathway.

To rule out the possibility that these inhibitors reduced proteolysis by some nonspecific toxic effect (e.g., inhibition of ATP synthesis could lead to the decrease in ATP-dependent proteolysis), we measured their effects on cell growth. Growth (as measured by OD_{600} or by number of colonies) was not affected by treatment with MG132 or β-lactone at least for 5 h (data not shown). This continued increase in cell numbers for 5 h was not

The Inhibition of the Ubiquitin-Proteasome Pathway—Most short-lived proteins, such as highly abnormal proteins, are believed to be degraded by the ubiquitin-proteasome pathway (4). To test whether these inhibitors also block the rapid breakdown of a model substrate of this pathway, we transfected ise1 cells with a plasmid carrying the abnormal fusion polypeptide, Ub-P-β-gal, and measured its degradation using pulse-chase and immunoprecipitation analysis (19, 24). In accord with prior data (24), this polypeptide had a half-life of 10 min in untreated ise1 cells (Fig. 3A), but in the presence of MG132 (at 50 μM) or β-lactone (20 μM), the breakdown of Ub-P-β-gal was dramatically inhibited. By contrast, neither MG132 nor β-lactone (90-min pretreatment) reduced synthesis of this protein (Fig. 3A).

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anticipated in light of the importance of ubiquitin-proteasome pathway in cell cycle regulation. However, proteolysis was inhibited at most by 70–80% under these condition (Fig. 1). Also the overall rate of incorporation of $[^{14}\text{C}]$leucine (data not shown) or $[^{35}\text{S}]$methionine (Fig. 3A) into cell proteins was not affected by these inhibitors. Thus the inhibition of proteolysis can not be due to ATP depletion, and the proteasome functions can be blocked for several hours without obvious deleterious effects on essential cell processes.

**Effect of Proteasome Inhibitors and PMSF on Degradation of Long-lived Proteins**—In mammalian cells, the breakdown of a significant fraction (10–30%) of long-lived cell proteins, which comprise the bulk of proteins, can be blocked by inhibitors of lysosomes, and this fraction increases in serum-deprived cells (25). However, recent studies with peptide aldehyde inhibitors indicate that the breakdown of most (70–90%) of the long-lived cell proteins is mediated by proteasomes (8, 26). These findings were surprising, because the ubiquitin-proteasome pathway had been generally thought to degrade primarily short-lived proteins, while the bulk of long-lived components were thought to be degraded by the lysosomal/vacuolar pathway by autophagic mechanism (27).

Experiments were undertaken to test whether proteasome also catalyzes the degradation of long-lived proteins in yeast. In contrast to finding in mammalian cells, MG132 (at 50 $\mu$m) did not inhibit the degradation of the long-lived proteins in *ise1* strain (Fig. 4A), although it reduced the degradation of short-lived proteins by more than 50% at the same concentration (Fig. 1B). Thus in yeast the long-lived components do not appear to be degraded by the proteasome.

PMSF is an inhibitor of serine proteases that blocks a number of yeast vacuolar proteases, such as proteinase B and carboxypeptidase Y (28), but does not affect proteasome function (29). Moreover, PMSF has been reported to block the degradation of proteins that accumulate in autophagic bodies in yeast cell (30). To test if PMSF influences generally the degradation of long-lived proteins, we incubated *ise1* cells with PMSF and then measured the degradation of different classes of cell proteins. PMSF (1 $\mu$m) inhibited the hydrolysis of long-lived proteins (50% inhibition), but did not reduce the rapid degradation of short-lived proteins (Fig. 4B). Thus the vacuolar system catalyzes the hydrolysis of long-lived proteins in exponentially growing yeast cells. Moreover, these two distinct degradation systems can be selectively inhibited by the use of these different types of inhibitors.

**DISCUSSION**

The functions of the ubiquitin-proteasome pathway have been extensively studied in yeast because of the availability of mutants affecting ubiquitin conjugation and proteasome subunits (3, 7, 31, 32). Often, however, analysis of these strains is complicated because of the pleiotropic effects of these mutations or second site suppressor mutations (e.g. cell compensation to reduce proteolysis). The availability of rapidly acting and reversible inhibitors (e.g. MG132) of the ubiquitin-proteasome pathway should be very useful for such studies. The degradation of a model substrate for ubiquitin pathway, for example, was dramatically inhibited by these agents, and most significantly, this polypeptide accumulated in an enzymatically active form (Fig. 3). By stabilizing the short-lived proteins, these agents also can provide an easy mean to increase the yield of otherwise unstable proteins in yeast cells.

Interestingly the proteasome inhibitors were effective only in a yeast mutant (*ise1*), apparently because they failed to penetrate wild-type yeast cells, even though all these inhibitors can penetrate mammalian cells readily. In initial experiments, we were also unsuccessful in demonstrating effects of these inhibitors in spheroplast prepared from wild-type cells. The mutation in *ise1* strain is allelic to the *erg6* mutation, which is defective in the biosynthesis of the principal membrane sterol of yeast, ergosterol (23). Either the change in the composition of the plasma membrane renders this strain more permeable to many drugs, or a transport system used to detoxify the yeast cell cannot function properly in the altered membrane (23).

It is noteworthy that lactacystin did not show any effect in *ise1* cells, although it could block the activity of proteasomes purified from wild-type yeast cells. On the other hand, $\beta$-lactone, the product of lactacystin hydrolysis (14), blocked the degradation of both short-lived and abnormal proteins to a similar extent as MG132. Recent studies showed that only $\beta$-lactone readily enters the cultured mammalian cells (lactacystin is relatively impermeable), and the conversion of lactacystin to $\beta$-lactone is rate-limiting for its inhibitory effects. Apparently, in the yeast growth medium, this conversion must be much slower than in mammalian tissue culture medium. Also higher levels of the $\beta$-lactone were necessary for inhibition in the yeast.

The reversible effect of the peptide aldehydes shown here is consistent with their mode of action and findings in mammalian cells (8). The treated cells could have not been damaged even by prolonged exposure to these inhibitors since they recovered fully their ability to degrade proteins. In addition the growth of cells was not affected by these inhibitors for at least 5 h. This continued growth was unexpected, since progression through certain stages of the cell cycle requires ubiquitin-de-

2 D. H. Lee and A. L. Goldberg, unpublished results.
3 L. R. Dick, A. A. Cruikshank, A. T. Destree, L. Grenier, T. A. McCormack, F. D. Melandri, S. L. Nunes, V. J. Palombella, L. A. Parent, L. Plamondon, and R. L. Stein, submitted for publication.
pendent degradation of cyclins and other regulatory proteins (6). Possibly the residual activity of the proteasome (20–30% of full activity) under these conditions is sufficient for degradation of these proteins. In fact, it is interesting that a major reduction in overall proteolysis can occur without preventing cell division for several hours. The increase in β-galactosidase activity of Ub-P-β-gal also clearly indicates that protein synthesis and folding can function, while the degradation of short-lived proteins is blocked. Because of their reversible and selective effects, the peptide aldehydes should be very useful for many experimental studies and perhaps also for industrial applications to increase the yield of short-lived regulatory proteins or unstable engineered proteins. One unexpected finding was that the inhibitory effect of the peptide aldehydes was lost after 4–5 h of incubation with yeast cells, which can inactivate the inhibitors, perhaps by oxidizing or by cleaving peptide bonds.

The lysosomal/vacuolar pathway has long been believed to be the major site for degradation of bulk of cell proteins by autophagic vacuole formation (27). The recent findings that proteasomes also catalyze the degradation of these long-lived components in cultured lymphoblasts, fibroblasts (8), as well as in skeletal muscle (26), are clearly in contrast with our present findings in yeast. In contrast to the peptide aldehydes and β-lactone, PMSF, which inactivates many vacuolar serine proteases (28) and prevents autophagic body formation (30) in yeast, was an effective inhibitor of the degradation of long-lived proteins. Thus, the vacuoles in yeast and not the proteasomes seem to play a primary role in the turnover of long-lived proteins. However, the relative importance of the proteasome and vacuolar system in overall protein degradation may vary in different cell types and under different physiological conditions. In any case, the yeast vacuole is clearly not the functional equivalent of the lysosomes in mammalian cells.

Since these protease inhibitors can differentially and selectively block either the ubiquitin-proteasome pathway or vacuolar proteolysis without obvious effects on other important cellular processes, they should greatly facilitate studies of the functions and modes of regulation of these two proteolytic systems in intact yeast.

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