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Ump1p Is Required for Proper Maturation of the 20S Proteasome and Becomes Its Substrate upon Completion of the Assembly

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Summary

We report the discovery of a short-lived chaperone that is required for the correct maturation of the eukaryotic 20S proteasome and is destroyed at a specific stage of the assembly process. The *S. cerevisiae* Ump1p protein is a component of proteasome precursor complexes containing unprocessed β subunits but is not detected in the mature 20S proteasome. Upon the association of two precursor complexes, Ump1p is encased and is rapidly degraded after the proteolytic sites in the interior of the nascent proteasome are activated. Cells lacking Ump1p exhibit a lack of coordination between the processing of β subunits and proteasome assembly, resulting in functionally impaired proteasomes. We also show that the propeptide of the Pre2p/Doa3p β subunit is required for Ump1p's function in proteasome maturation.

Introduction

Ubiquitin (Ub)–dependent proteolysis underlies the bulk of nonlysosomal protein degradation in eukaryotic cells (reviewed by Hochstrasser, 1996; Varshavsky, 1997). Naturally short-lived as well as damaged or otherwise abnormal proteins are recognized by the Ub system and are marked for degradation by the attachment of multi-Ub chains. Ubiquitylated proteins are degraded by the 26S proteasome, an ~2000 kDa, multisubunit, ATP-dependent protease that consists of the 19S complex, which is required specifically for the degradation of ubiquitylated proteins, and an ~700 kDa complex, called the 20S proteasome, which is the ATP-independent catalytic core of the 26S proteasome (reviewed by Peters, 1994; Coux et al., 1996; Lupas et al. 1997).

The 20S proteasome is universal among eukaryotes; its structural homologs have also been found in archaeons and eubacteria (reviewed by Coux et al., 1996). High-resolution crystal structures have been reported for the 20S proteasomes of the archaeon *Thermoplasma acidophilum* and the eukaryote *Saccharomyces cerevisiae* (Löwe et al., 1995; Groll et al., 1997). The *Thermoplasma* proteasome contains two types of subunits, α and β, which form a hollow cylinder composed of four heptameric rings in the configuration $\alpha_7\beta_7\beta_7\alpha_7$. The yeast 20S proteasome has a similar structure but contains 14 distinct subunits, seven of the α type and seven of the β type. The N-terminal threonines of the β subunits of the *Thermoplasma* proteasome act as nucleophiles in catalyzing the hydrolysis of peptide bonds of polypeptide substrates (Seemüller et al., 1995). Lys-33 and Glu-17 of the β subunit also play a role in catalyzing the cleavage of peptide bonds. The 14 identical β subunits of the *Thermoplasma* proteasome form 14 identical active sites, which catalyze the cleavage of substrates after hydrophobic amino acid residues (chymotrypsin-like active sites). These sites are located on the inner surface of a central chamber formed by the two rings of β subunits (Löwe et al., 1995; Seemüller et al., 1995). β subunits are synthesized as inactive precursors containing a propeptide that is thought to be cleaved off autocatalytically, yielding the mature β subunit bearing N-terminal Thr. The propeptides of β subunits are not required for the in vitro assembly of the *Thermoplasma* proteasome (Seemüller et al., 1996).

In eukaryotic proteasomes, only 3 of the 7 distinct β subunits contain the three conserved residues required for activity of the *Thermoplasma* proteasome. Genetic and structural data suggest that these three subunits provide the active-site nucleophiles for the three distinct catalytic activities of eukaryotic proteasomes, namely the "chymotrypsin-like" (see above), the "trypsin-like" (cleavage after basic residues), and the "peptidylglutamyl peptide hydrolyzing (PGPH)" activity (cleavage after acidic residues). In *S. cerevisiae*, these three β subunits are Pre2p/Doa3p, Pup1p, and Pre3p (Heinemeyer et al., 1993; Chen and Hochstrasser, 1996; Arendt and Hochstrasser, 1997; Groll et al., 1997; Heinemeyer et al., 1997). In the human 20S proteasome, the related proteins are MB1, δ, and Z; during the immune response, these subunits can be replaced by their respective homologs LMP7, LMP2, and MECL-1/LMP10 (reviewed by Coux et al., 1996). In the case of LMP2 and LMP7, it has been demonstrated that these replacements are functionally relevant in altering the specificity of antigen presentation by the MHC class I pathway. The catalytically active β subunits of the eukaryotic proteasome are synthesized with propeptides, similarly to the β subunit in *Thermoplasma* (Schmidtke et al., 1997).

In the crystal structure of the *S. cerevisiae* 20S proteasome, the opening to the proteasome's interior, formed by the outer ring of the α subunits, is not large enough to admit even an unfolded polypeptide chain, let alone a folded protein (Groll et al., 1997). The degradation of larger substrates requires the 19S complexes, which attach at both sides of the 20S proteasome, yielding the 26S proteasome. The 19S complexes contain subunits that bind multi-Ub chains, at least one Ub-specific isopeptidase that disassembles these chains, and several

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ATPases that are thought to be involved in perturbing the substrate conformationally and guiding it to the interior of the 20S proteasome (Deveraux et al., 1994; Hochstrasser et al., 1995; Jentsch and Schlenker, 1995; Coux et al., 1996).

Studies with mammalian cells have shown that the 20S proteasome is assembled through a 15–16S intermediate, apparently a half-proteasome. This intermediate contains all 14 α and β subunits, some of which are in the precursor (propeptide-bearing) form, and several uncharacterized polypeptides as well (Frentzel et al., 1994; Yang et al., 1995; Nandi et al., 1997; Schmidtke et al., 1997). Studies on the yeast 20S proteasome have demonstrated that processing of proPre2p (identical to proDoa3p) is coupled to formation of the 20S proteasome from two half-proteasome precursors (Chen and Hochstrasser, 1996). Formation of the active site capable of autocatalytic processing of proPre2p depends on the juxtaposition of proPre2p and Pre1p on the opposite sides of the two halves of the proteasome. This mechanism is thought to prevent activation of proteolytic sites before the central hydrolytic chamber has been sealed off from the cytosol through association of the two halves of the proteasome (Chen and Hochstrasser, 1996). The Pre2p propeptide is essential for the formation of functional proteasomes. Moreover, this propeptide can operate in *trans*, suggesting that it serves a chaperone-like function in proteasome maturation (Chen and Hochstrasser, 1996).

In the present work, we identify Ump1p, a novel protein, as a component of a precursor complex of the 20S proteasome. This precursor contains unprocessed β subunits. Upon formation of the 20S proteasome from two such precursors, the propeptides of β subunits are removed, a process that is accompanied by removal of Ump1p from the proteasome through Ump1p degradation, which requires the proteasome's proteolytic activity. In *ump1*Δ cells, which lack Ump1p, coordination of 20S complex formation and processing of β subunits is impaired, resulting in incompletely or (in the case of Pre2p) prematurely processed β subunits. These findings reveal Ump1p as a novel type of molecular chaperone, a short-lived maturation factor required for the efficient biogenesis of the 20S proteasome. We also describe genetic evidence that the propeptide of proPre2p is required for Ump1p-dependent proteasome maturation, and we present a model that accounts for some of the functions of Ump1p and propeptides in proteasome maturation.

Results

The *UMP1* Gene Is Required for Ubiquitin-Mediated Proteolysis

To identify the genes required for Ub-dependent proteolysis in *S. cerevisiae*, we screened for mutants defective in the degradation of test substrates. One such mutant, termed *ump1-1* (**u**b-**m**ediated **p**roteolysis), exhibited defects in the degradation of several normally short-lived proteins (see below). The complementing *UMP1* gene encoded a 148-residue (16.8 kDa) protein. Searches of the databases did not identify close homologs of Ump1p but did detect similarities to regions of several proteins. One intriguing similarity was between Ump1p and C-terminal regions of the protease inhibitor contrapsin (25% identity, 52% similarity) and related proteins (Figure 1A), consistent with the likely role of Ump1p as an inhibitor of premature proteolytic activation of the proteasome precursor complexes (see below).

Pulse-chase analyses in *ump1*Δ mutants revealed a strong stabilization of several normally short-lived test proteins, in particular an N-end rule substrate Arg–β-galactosidase (Arg–β-gal), which bears N-terminal Arg, a destabilizing residue (Varshavsky, 1996), Ub-Pro–β-gal (a substrate of the UFD pathway; Johnson et al., 1995; Varshavsky, 1997), and $\alpha 2_{deg1}$–β-gal (a substrate of the DOA pathway; Hochstrasser and Varshavsky, 1990; Hochstrasser et al., 1995) (Figure 1B). The recognition and ubiquitylation of these substrates involve different recognins (E3 proteins) and different Ub-conjugating (E2) enzymes. The pleiotropic character of the *ump1*Δ phenotype suggested that Ump1p functions downstream of the recognition and ubiquitylation components of the Ub system, perhaps at the step of proteolysis, or in regulating the supply of Ub.

*ump1*Δ Mutants Are Hypersensitive to a Variety of Stresses, Accumulate Ub-Protein Conjugates, and Do Not Sporulate

*ump1*Δ mutants grew more slowly than congenic wild-type (wt) cells at 30°C or lower temperatures and were severely growth-impaired at higher temperatures (37°C) (Figure 1C and data not shown). In addition, they were hypersensitive to cadmium ions and to the arginine analog canavanine (Figure 1C). Similar phenotypes have been reported for mutants in genes encoding E2 (Ubc) enzymes or proteasome subunits (Heinemeyer et al., 1993; Jungmann et al., 1993). Comparisons of proteins from whole-cell extracts of wt and *ump1*Δ cells by immunoblotting with anti-Ub antibodies showed a dramatic accumulation of Ub-protein conjugates in the *ump1*Δ mutant. At the same time, the level of free Ub was reduced in *ump1*Δ cells (data not shown). Thus, the primary cause of the *ump1*Δ phenotype appears to be the impaired ability of *ump1*Δ cells to degrade Ub-protein conjugates. Homozygous *ump1*Δ diploids (strain JD61) were unable to sporulate (data not shown).

Ump1p Is a Component of Proteasome Precursors

To investigate Ump1p biochemically, an epitope tag was linked to its C terminus. A single copy of the modified *UMP1* gene (*UMP1-ha*), expressed from its natural promoter and chromosomal location, restored wt growth rates. We analyzed whole-cell extracts of a strain expressing Ump1p-ha and Pre1p-ha (the latter an ha-tagged β subunit of the proteasome) by gel filtration on Superose-6. Ump1p-ha eluted in fractions 22 and 23, both of which also contained Pre1p-ha and other proteasomal subunits (Figures 2A, 2B, and 3A) but lacked the chymotrypsin-like activity of the mature 20S proteasome. The apparent size of the Ump1p-containing complex was 300–400 kDa (Figure 2A). 20S and 26S proteasomes, as well as free 19S caps, and 20S proteasomes with one attached 19S cap, eluted in earlier fractions

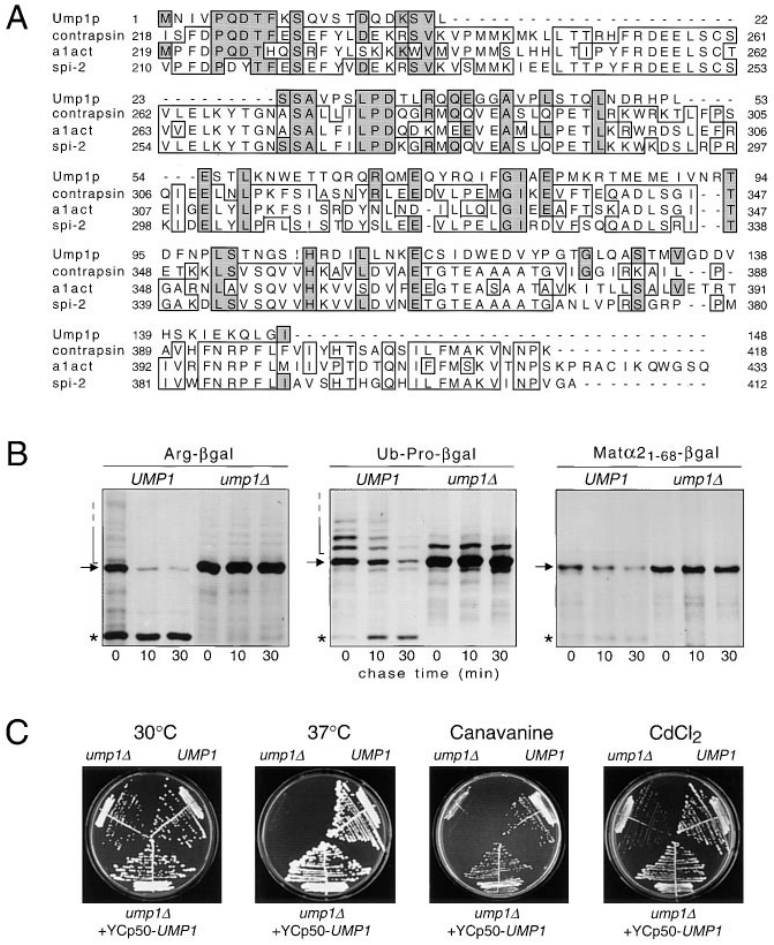

Figure 1. Ump1p Is Required for Ubiquitin-Mediated Proteolysis

(A) Sequence similarities between Ump1p and C-terminal parts of mouse contrapsin, human alpha1 antitrypsin inhibitor (a1act) and mouse spi-2 (PIR accession numbers, respectively, JX0129, A90475, and S31305). The sequences were aligned using the PileUp program (GCG package, version 7.2, Genetics Computer Group, Madison, WI). Gaps (indicated by hyphens) were used to maximize alignments. Residues identical between Ump1p and at least one of the other proteins are shaded in gray. Residues identical among at least two proteins other than Ump1p are boxed.

(B) Pulse-chase analysis comparing the metabolic stabilities of R–β-gal, Ub-P–β-gal, and MATα2$_{1-68}$–β-gal in wt (*UMP1*) and *ump1*Δ cells. The open-ended brackets denote the position of multiubiquitylated β-gal species. Asterisks denote an ~90 kDa β-gal cleavage product characteristic of short-lived β-gal derivatives (Dohmen et al., 1991).

(C) Growth of *ump1*Δ (JD59), *UMP1* (JD47-13C), and *ump1*Δ cells transformed with YCp50-*UMP1* (expressing wild-type *UMP1*). Cells were streaked on YPD plates and incubated for 2 days at 30°C (or at 37°C where indicated), with 0.8 μg/ml canavanine or 30 μm $CdCl_2$ where indicated.

(15–21), as determined by nondenaturing gel electrophoresis (Figure 2B and data not shown), by assays of the proteasome's proteolytic activity, and by immunological detection of Cim3p, a subunit specific for the 19S cap of the 26S proteasome (Ghislain et al., 1993).

Cochromatography of Ump1-ha and Pre1p-ha in fractions 22 and 23 (Figure 2A) suggested that Ump1p is a component of proteasome precursor complexes. Consistent with this possibility, the Ump1p-containing complex had a higher mobility than the 20S proteasome upon nondenaturing gel electrophoresis. A proteasome precursor with an electrophoretic mobility indistinguishable from that of the Ump1p-containing complex was also detected in extracts from a strain that expressed Pup1p-ha, another β subunit of the 20S proteasome (Figure 2B).

To produce independent evidence bearing on the nature of the Ump1p-containing complex, we constructed a strain that expressed both Ump1p-ha and doubly tagged Pre1p-Flag-His$_6$ (Pre1p-FH). Affinity chromatography on Ni-NTA-agarose and anti-Flag antibody resin was then used to purify complexes containing Pre1p-FH. Ump1p-ha cofractionated with Pre1p-FH in both affinity purification steps (Figures 2C and 2D), confirming that Ump1p is a component of a distinct proteasome-related complex. This complex sedimented at ~15S in sucrose gradients (data not shown). Taken together, this evidence strongly suggested that the Ump1p-containing complex is a precursor of the 20S proteasome that is similar to the 15–16S precursors observed in the maturation pathway of mammalian proteasomes (Frentzel et al., 1994; Yang et al., 1995).

The Ump1p Proteasome Precursor Complex Contains Unprocessed β Subunits

Several subunits of the proteasome are synthesized as precursors containing N-terminal extensions (propeptides) that are absent from the mature proteasome. These precursors are detected in the mammalian 15–16S complex (Frentzel et al., 1994; Yang et al., 1995). To compare the Ump1p-containing complex with the mammalian 15–16S complex, we analyzed the former for the presence of propeptide-containing proteasomal subunits. These β subunits were modified by the addition of C-terminal ha tags. Strains expressing Pup1p-ha, Pre2p-ha, and Pre3p-ha grew at wt rates (data not shown). When extracts of these strains were fractionated by gel filtration on Superose-6, the fractions containing the Ump1p complex contained largely the β subunit precursors, proPup1-ha, proPre2p-ha, and proPre3p-ha (Figure 3A). The corresponding mature β subunits largely eluted in the earlier fractions that contained 20S and 26S proteasomes (Figure 3A). Subtle but reproducible differences

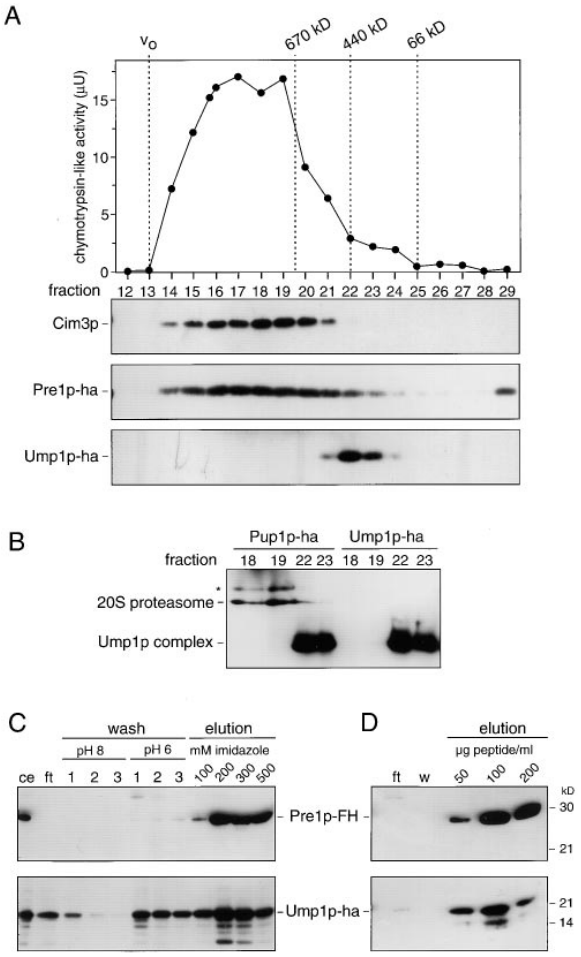

Figure 2. Ump1p Is a Component of Proteasome Precursor Complexes

(A) An extract from strain JD127 expressing Ump1p-ha and Pre1p-ha instead of wt versions of these proteins was fractionated by gel filtration on Superose-6. The upper panel shows the results of measurements of chymotrypsin-like activity in the relevant fractions (12–29). The last fraction, representing the void volume (v_o), and positions of the peaks of marker proteins are indicated by dashed lines. The same fractions were analyzed by SDS-PAGE and immunoblotting with anti-Cim3p and anti-ha antibodies, which detected Cim3p, Pre1p-ha, and Ump1p-ha, as indicated.

(B) Analysis of Superose-fractionated 20S proteasomes and precursor complexes by nondenaturing gel electrophoresis and immunoblotting with anti-ha antibody. The strains were JD139 and JD129 expressing, respectively, Pup1p-ha and Ump1p-ha. Asterisk, 20S proteasome with one attached 19S regulator cap. The 26S proteasome eluted in fractions 14–17 of Superose-6 (not shown).

(C) Affinity purification of Pre1p-FH on Ni-(NTA)-Sepharose and copurification of Ump1p-ha. Purification of proteins from an extract of strain JD126 expressing Ump1p-ha and Pre1p-FH was carried out as described in the Experimental Procedures. Different fractions were analyzed by SDS-PAGE and immunoblotting with anti-Flag (upper panel) and anti-ha antibodies (lower panel). Ce, crude extract; ft, flow-through. In contrast, no binding of Ump1p to the resin was observed when extracts from strain JD129 expressing untagged Pre1p were used (not shown).

(D) Affinity purification of Pre1p-FH on anti-Flag antibody agarose resin. Material that was eluted from the Ni-(NTA)-Sepharose column shown in panel C was subjected to a second affinity purification on anti-Flag antibody resin. Pre1p-FH was specifically eluted with increasing concentrations of the Flag peptide. W, wash.

were detected among the tagged β subunits with respect to the distribution of their precursor and processed forms in various fractions. For example, pro-Pup1p-ha was detectable only in fractions 22 and 23, and was absent from fraction 21. By contrast, proPre2p-ha was detectable in the larger complexes (down to fraction 19), while fractions 22 and 23 already contained some processed Pre3p-ha. These patterns suggested a defined order of processing events. One conclusion from these experiments is that the Ump1p-containing complex is a precursor of the 20S proteasome that contains unprocessed β subunits and is therefore proteolytically inactive (Figures 3A and 3C).

Ump1p Is Required for Correct Proteasome Maturation

Next, we asked what effect the *ump1*Δ mutation has on maturation and activity of the proteasome. Specifically, the analyses described above were repeated with extracts obtained from *ump1*Δ cells. Figure 3C shows that there was a significant reduction in the three proteolytic activities of the proteasome in the fractions containing the 20S and 26S proteasomes (fractions 14–21). The reduction of the specific activity appears to be partially compensated by an increased expression of proteasomes (Figures 3A and 3B, data not shown) similar to that observed in cells expressing mutant β subunits (Arendt and Hochstrasser, 1997). In addition, a new (absent from wt cells) peak of chymotrypsin-like activity encompassing fractions 22 and 23 was detected in extracts from *ump1*Δ cells. These were the fractions that contained the Ump1p complex from wt extracts (see above and Figure 3A), suggesting that the absence of Ump1p from the proteasome precursor complex was the cause for premature activation of its chymotryptic activity in the *ump1*Δ mutant. To verify that the detected activity was actually of the ~15S precursor complex from *ump1*Δ cells, the fraction 22 samples from strains expressing either wt Pre2p or Pre2p-ha were incubated with anti-ha antibody and protein A Sepharose. This treatment did not deplete the chymotryptic activity from the sample containing untagged Pre2p but did deplete ~80% of the activity from an otherwise identical sample derived from *ump1*Δ cells expressing Pre2p-ha (data not shown). Thus, the chymotryptic activity of this fraction resided in the proteasome precursor complexes.

To follow the appearance of the precursor and mature forms of β subunits in proteasome precursors and mature proteasomes, we analyzed extracts from *ump1*Δ cells expressing ha-tagged versions of these subunits by immunoblotting with anti-ha antibody. The processing of the three analyzed β subunits, Pup1p-ha, Pre2p-ha, and Pre3p-ha, was strikingly different in *ump1*Δ and wt cells (Figure 3A). For all three subunits, a dramatic increase of their precursors was detected in the fractions (14–21) that contained the 20S and 26S forms of the proteasome. In addition, different processed variants, possibly representing processing intermediates of pro-Pre2p, could be detected in fractions 22 and 23, which contained the ~15S proteasome precursor, and in some of the proteasome-containing fractions as well (Figure 3A). In contrast, with the exception of Pre3p, almost no processed β subunits were present in the fractions

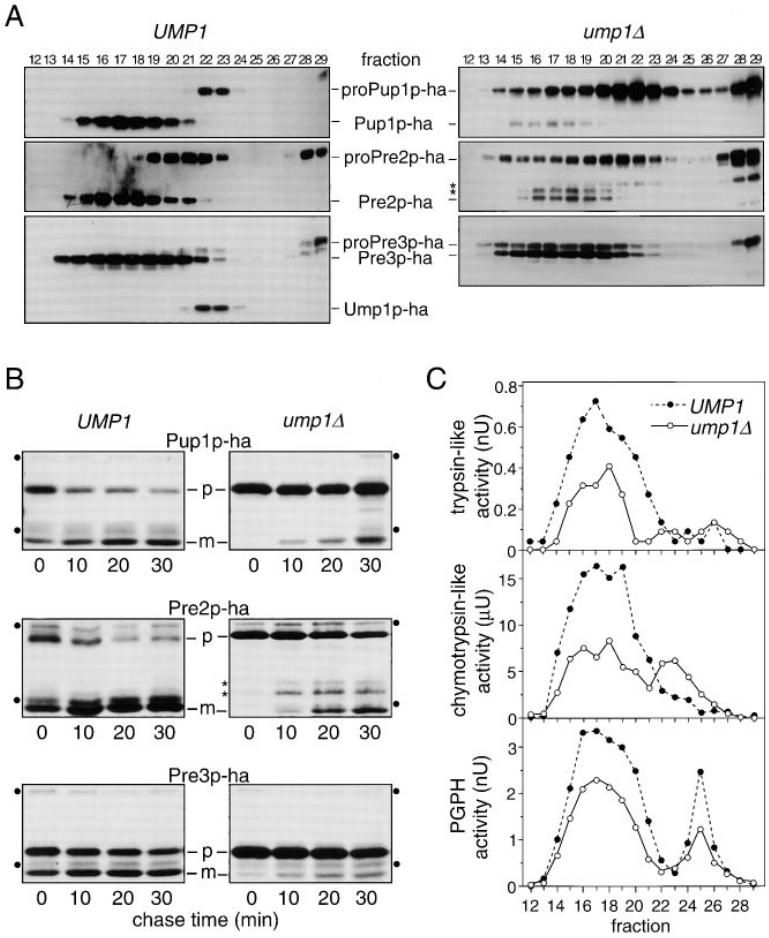

Figure 3. Ump1p Is Required for the Correct Processing of Proteasomal β Subunits

(A) SDS-PAGE and anti-ha immunoblot analyses of extracts fractionated on Superose-6. The congenic yeast strains used were JD131, JD132, JD133, JD134, JD135, and JD136 (Table 1). Western blots of the *ump1*Δ extracts were developed for a shorter time to compensate for an increased expression of proteasomal subunits in the mutant. Asterisks, processing intermediates or incompletely processed forms of Pre2p-ha that were observed in the *ump1*Δ mutant but not in wt cells.

(B) Pulse-chase analysis comparing the rate of β subunit processing in wt cells to that in *ump1*Δ cells (same strains as in A). Cells were pulse-labeled with ^{35}S-Met/Cys for 5 min. Proteins were precipitated with anti-ha antibody. Fluorographic exposures were ~3 times longer for the *UMP1* samples. P, propeptide-containing precursor form; m, mature form; asterisks, as in (A); dots, nonspecific bands present in some samples.

(C) Comparison of proteolytic activities in extracts with equal amounts of total protein of *UMP1* (JD127) and *ump1*Δ cells (JD75) fractionated by gel filtration on Superose-6. The profile of the chymotrypsin-like activity in *UMP1* is the same as in Figure 2A.

containing the ~15S proteasome precursor from wt cells, and no processing intermediates of Pre2p could be detected either (Figure 3A). These findings indicate that Ump1p is required to prevent premature processing of at least proPre2p and that the presence of Ump1p is important for the coordination of proteasome assembly and subunit processing.

A defect of *ump1*Δ cells in the processing of β subunits was also observed by following the metabolic fate of these subunits in pulse-chase experiments. In wt cells, most of the propeptide-containing forms of β subunits (proPup1-ha, proPre2p-ha and proPre3p-ha) were converted into their mature counterparts during the 30-min chase (Figure 3B). By contrast, in the *ump1*Δ mutant, the bulk of the β subunit precursors remained unprocessed during this time, a finding consistent with the results of immunoblot analyses (Figure 3A). In the case of Pre2p-ha, pulse-chase analysis again revealed the species of intermediate size (putative processing intermediates) in extracts from *ump1*Δ cells. These species were absent from the equivalent samples derived from wt cells (Figure 3B).

Ump1p Is Degraded upon Formation of the 20S Proteasome

The experiments above demonstrated that Ump1p is a component of proteasome precursors that is absent from the mature 20S proteasome. One possibility was that Ump1p leaves the precursor complex upon formation of the 20S proteasome from the two ~15S precursors and may then assist with another round of proteasome assembly, thus acting catalytically. It was also possible that formation of the 20S proteasome sterically traps Ump1p. Since the formation of the 20S form of the proteasome coincides with the appearance of its proteolytic activities (Frentzel et al., 1994; Chen and Hochstrasser, 1996), Ump1p might be degraded by the newly formed 20S proteasome. Pulse-chase analysis of Ump1p-ha in wt cells showed that Ump1p-ha is indeed degraded in vivo, the rate of its degradation being similar to the rate of disappearance of proPup1p-ha, which is converted to Pup1p-ha upon maturation of the proteasome (compare Figures 4A and 3B).

Ump1p Is Stabilized in Proteasome Mutants and Can Be Detected Inside the 20S Particle

If Ump1p becomes a substrate of the newly formed 20S proteasome, the degradation of Ump1p should be inhibited by mutations that affect the proteasome's proteolytic activities. We used pulse-chase assays to follow the metabolic fate of Ump1p-ha in the *pre1-1* mutant, which is known to be deficient in the chymotryptic activity of the proteasome (Heinemeyer et al., 1993). Ump1p was partially stabilized in *pre1-1* cells, as indicated by the increased amount of ^{35}S-labeled Ump1p in *pre1-1*

extracts versus wt extracts that contained the same total amount of TCA-precipitable ^{35}S (Figure 4A). However, we still observed a significant decrease of pulse-labeled Ump1p upon increasing chase times when the immunoprecipitation was carried out under nondenaturing conditions (Figure 4A). By contrast, when the extracts were treated with 0.4% SDS at 100°C before immunoprecipitation, virtually no decrease of the Ump1p signal during the chase was observed in *pre1-1* cells. These results were consistent with the possibility that the newly formed Ump1p-ha became inaccessible to the anti-ha antibody during the chase because it became trapped within the newly formed *pre1-1* proteasome. If so, Ump1p was expected to be present in fractions from the Superose-6 column that corresponded to the 20S and 26S proteasomes in the *pre1-1* mutant. Indeed, whereas in wt (*PRE1*) cells Ump1p-ha was detected by SDS-PAGE and immunoblotting only in fractions 22 and 23 (corresponding to the ~15S proteasome precursor complex), in the mutant (*pre1-1*) cells Ump1p-ha was detected in fractions 15–23, indicating that it was also present in mature proteasomes (Figure 4B). We assayed the accessibility of Ump1p-ha to anti-ha antibody by immunoprecipitation from fraction 19 (the 20S proteasome) of extracts from the *pre1-1* mutant and from fraction 22 (the ~15S proteasome precursor) of extract from *PRE1* cells. Ump1p-ha could be immunoprecipitated from either fraction 19 or 22 following pretreatment with 0.1% SDS, but not under nondenaturing conditions (Figure 4C), indicating that the ha tag was inaccessible to the anti-ha antibody in both the *pre1-1* proteasome and the precursor complex. However, when otherwise identical assays were carried out with a polyclonal anti-Ump1p antiserum, most of Ump1p could be immunoprecipitated under nondenaturing conditions from fraction 22 (the ~15S proteasome precursor) but was still not immunoprecipitated from fraction 19 (the 20S *pre1-1* proteasome). In contrast, when polyclonal anti-proteasome antibodies were used, Ump1p was precipitated quantitatively along with both complexes (Figure 4C).

These results indicated that a part of Ump1p other than its C terminus is accessible to anti-Ump1p antibodies in the ~15S precursor. However, Ump1p becomes entirely inaccessible (under nondenaturing conditions) upon formation of the 20S proteasome from two ~15S precursors. This interpretation was supported by examining the sensitivity of Ump1p-ha in different complexes to trypsin digestion (Figure 4D). Specifically, Ump1p-ha in the 20S proteasomes (fraction 19) from *pre1-1* cells was completely protected against trypsin. In contrast, Ump1p-ha in the ~15S proteasome precursors was detectably accessible to trypsin. The degradation of Ump1p was incomplete, resulting in a protected fragment of Ump1p-ha that lacked the ~5 kDa N-terminal region but retained the C-terminal ha tag (Figure 4D). Interestingly, in a similar experiment with a strain expressing pro-Pre2p-ha, we observed that, in the Ump1-containing ~15S proteasome precursor complex (fraction 22), pro-Pre2p-ha was shortened by trypsin treatment to yield a product whose electrophoretic mobility was indistinguishable from that of the natural mature Pre2p-ha (Figure 4D). In these experiments, the overall structure of the Ump1p proteasome precursor complex remained intact as judged by native gel analysis of trypsin-treated material (data not shown).

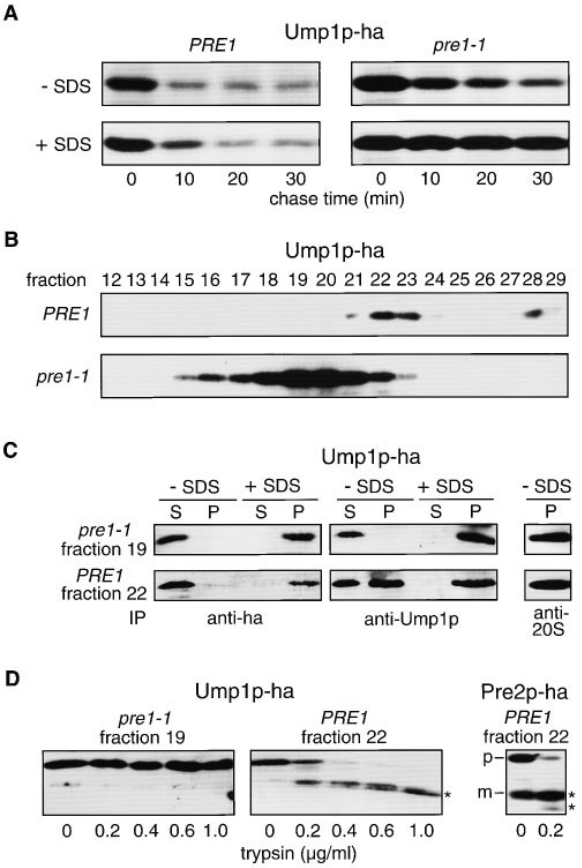

Figure 4. Ump1p Is Stabilized in *pre1-1* Mutants Cells that Are Defective in the Proteasome's Chymotrypsin-like Activity and Persists in 20S Proteasomes

(A) Pulse-chase analysis of Ump1p-ha in wt and *pre1-1* cells. The strains used were JD150 (*PRE1*) and JD151 (*pre1-1*) (Table 1), both expressing Ump1p-ha. The cells were pulse-labeled with ^{35}S-Met/Cys for 5 min. Extracts were prepared from samples taken at different chase times. Samples were then split in halves. One-half (−SDS) was subjected to immunoprecipitation with anti-ha antibody according to the standard pulse-chase protocol. The other half (+SDS) was adjusted to 0.4% SDS and incubated at 100°C for 5 min, then diluted with extraction buffer to the final concentration of 0.1% SDS prior to immunoprecipitation.

(B–D) SDS-PAGE and anti-ha immunoblot analyses. (B) Detection of Ump1p-ha-containing complexes in extracts fractionated by Superose-6 gel filtration. Strains were the same as in (A). Note the accumulation of Ump1p-ha in fractions containing the 20S and 26S proteasome (fractions 15–21) in *pre1-1*. (C) Immunoblot analyses of immunoprecipitations were carried out with material from Superose-6 fraction 19 of extracts from strain JD151 and fraction 22 of extracts from strain JD150, using the indicated antibodies. Immunoprecipitations were performed without addition of SDS (−SDS) or after boiling in the presence of 0.1% SDS (+SDS). P, precipitates; S, supernatants. (D) Assaying trypsin sensitivity of the same material as in (C), and of the Superose-6 fraction 22, from strain JD138 expressing Pre2p-ha. P, proPre2p-ha; m, mature Pre2p-ha; asterisks mark trypsin digestion products.

In the *ump1*Δ Mutant, the Propeptide of Pre2p Is Not Required for Incorporation of Pre2p into the Proteasome

Chen and Hochstrasser (1996) have elegantly demonstrated that the propeptide of Pre2p, if separated from the mature Pre2p, could function in *trans* and thereby

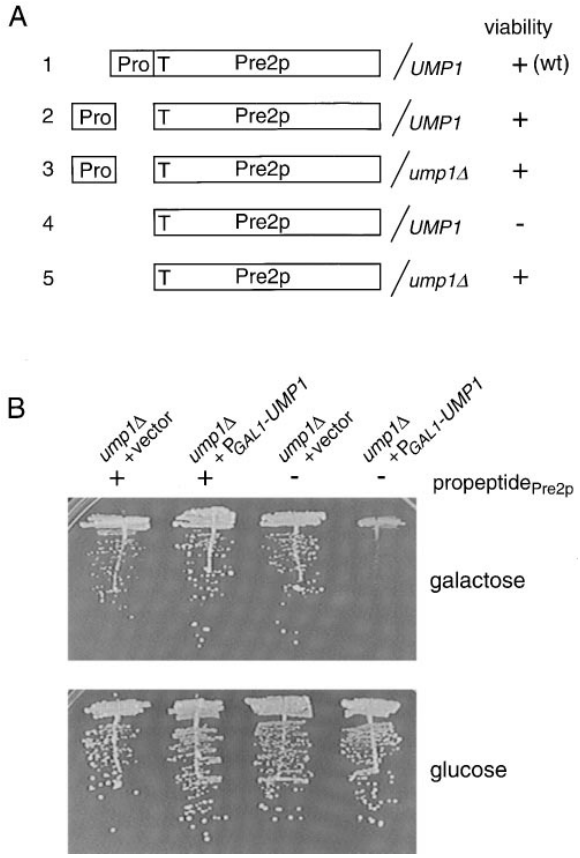

Figure 5. The Pre2p Propeptide Is Not Essential in the *ump*Δ Mutant

(A) Representation of different genotypes of a set of congenic strains and their effects on cell viability. Strain 1 (JD50-10B) is the wt control. Strain 2 (MHY952) expresses Pre2p/Doa3p-ΔLS with N-terminal Thr (T) as a fusion to ubiquitin (not shown), and the Pre2p/Doa3p propeptide (LS) from two separate plasmids (Chen and Hochstrasser, 1996). Strain 3 (JD160), same as strain 2 but *ump1*Δ. Strain 4 would be derived from strain 2 through the loss of the plasmid encoding Pre2p/Doa3p-LS that operates in *trans*. We were unable to produce isolates that lost this plasmid, confirming the earlier demonstration that the propeptide is essential for cell viability (Chen and Hochstrasser, 1996). Strain 5 (JD163) is derived from strain 3 through the loss of the propeptide-encoding plasmid. Loss of the plasmid did not affect cell viability in the *ump1*Δ background.

(B) Induction of *UMP1* expression in the *ump1*Δ background inhibits the growth of a strain that lacks the Pre2p propeptide. Strains 3 (plus) and 5 (minus) shown in (A) were transformed with pJD429 that expressed *UMP1* from the galactose-inducible, glucose-repressible P_{GAL1} promoter, or with an empty vector as a control. Transformants selected on glucose media were pregrown on selective media containing raffinose as a carbon source, then streaked onto selective media with 2% galactose or 2% glucose, and grown for 3 days at 30°C.

still allow the incorporation of Pre2p into the 20S proteasome. Under these conditions, expression of the propeptide is essential for cell viability, suggesting that the propeptide, in *cis* or at least in *trans*, is required for the assembly of an active proteasome (Chen and Hochstrasser, 1996).

We confirmed this result using their strain MHY952, in which mature Pre2p and the propeptide are expressed on separate plasmids (Figure 5A). Specifically, under nonselective growth conditions this strain did not yield viable cells that had lost the plasmid expressing the propeptide. Surprisingly, however, when we constructed and examined a congenic *ump1*Δ derivative of this strain, we noticed that it lost the propeptide-expressing plasmid at a frequency suggesting that this plasmid did not provide a significant growth advantage to cells (data not shown). Indeed, growth rates of cells with the plasmid were indistinguishable from those lacking it (Figure 5B). This result demonstrated that *ump1*Δ is a suppressor of a deletion of the propeptide of Pre2p. If so, expression of *UMP1* in the (*ump1*Δ *PRE2*-Δ*pro*) background should be incompatible with cell viability. This prediction was confirmed when we examined growth properties of the (*ump1*Δ *PRE2*-Δ*pro*) strain transformed with a plasmid expressing *UMP1* from the galactose-inducible P_{GAL1} promoter. On glucose-containing media, the growth rate of this transformant was indistinguishable from that of an otherwise identical transformant carrying the propeptide-expressing plasmid. In contrast, on galactose-containing media, the (*ump1*Δ *PRE2*-Δ*pro*) mutant containing the P_{GAL1}-*UMP1* plasmid was unable to grow, whereas the control strains grew (Figure 5B). This result indicated that the propeptide of Pre2p is essential for the Ump1p-assisted maturation of the proteasome but is not essential for (partially) defective maturation of the proteasome that takes place in the absence of Ump1p.

Discussion

Ump1p, a Novel Maturation Factor of the 20S Proteasome

We describe the discovery of a proteasome maturation factor, termed Ump1p, whose unique properties include a short in vivo half-life that is due to its degradation within the newly formed proteasome. Ump1p is required for coordination of the proteasome's physical assembly and enzymatic activation. In addition, the normally essential propeptide of the Pre2p β subunit was found to become nonessential in the absence of Ump1p. We report the following specific results.

ump1Δ *Mutants Are Defective in Ub-Mediated Proteolysis*

They are sensitive to a variety of stresses and accumulate Ub-protein conjugates (Figure 1 and data not shown). All of the observed phenotypes of *ump1*Δ cells are consistent with the conclusion that *ump1* mutants are impaired in proteasome biogenesis and, consequently, in the degradation of ubiquitylated proteins.

The UMP1 *Gene*

It encodes a polypeptide with a calculated molecular mass of 16.8 kDa. No close sequence homologs of Ump1p were detected in the current databases. However, the presence of a small protein similar in size to Ump1p in preparations of the half-proteasome precursors in mammals (Frentzel et al., 1994; Yang et al., 1995; Nandi et al., 1997; Schmidtke et al., 1997) suggests the presence of a functional homolog of Ump1p in the mammalian proteasome maturation pathway.

Ump1p Is a Component of Proteasome Precursors that Contain Unprocessed β *Subunits*

The Ump1p-containing complex has a molecular mass of 300–400 kDa, sediments at ~15S, migrates significantly faster in native gels than the 20S proteasome,

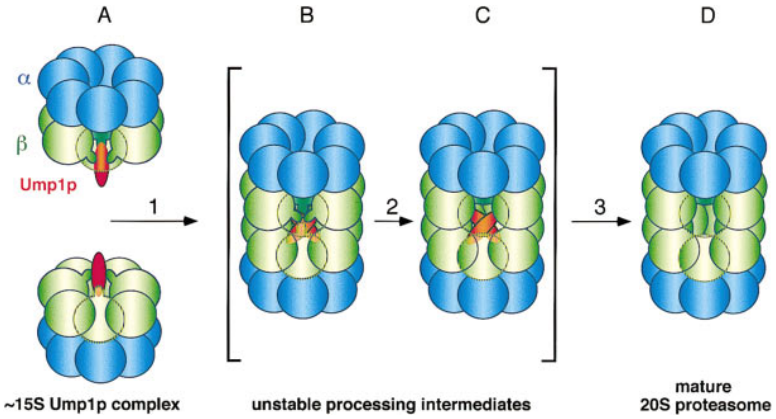

Figure 6. A Model of the Ump1p Function in Proteasome Maturation

Shown is a schematic view of the 20S proteasome and its precursor forms, with the α and β subunits as blue and green balls, respectively. Three of the β subunits are drawn with extensions that represent propeptides. The β subunits in the front are drawn transparent in order to allow a view into the interior chamber of the proteasome. Structure A is a proteasome precursor complex (half proteasome), characterized by the presence of Ump1p and unprocessed β subunits. In step 1, two of these precursors join to build structure B, a step that leads to conformational or positional shifts of Ump1p and propeptides. Conformational changes of the propeptides trigger their autocatalytic processing (step 2), and activation of the proteasome's proteolytic activities. This leads to the degradation of the chamber-entrapped Ump1p by the newly formed proteasome (step 3). Only structures A and D are long-lived enough to be detected in wt cells. The findings with the *pre1-1* mutant, in which the degradation of Ump1p is inhibited, suggest the existence of the short-lived intermediates B and C. See text for details.

and has a subunit composition that is highly similar to that of the 20S proteasome, as determined by SDS-PAGE and immunoblot analyses with anti-20S proteasome antibodies (Figure 2 and data not shown). Taken together, these results indicate that the Ump1p is contained within a half-proteasome precursor complex, whose counterpart has been described in the maturation pathway of mammalian proteasomes (Frentzel et al., 1994; Yang et al., 1995; Nandi et al., 1997; Schmidtke et al., 1997).

Maturation of Pup1p, Pre2p, and Pre3p Is Strongly Impaired in the ump1Δ *Mutant*

Considerably increased amounts of the unprocessed (propeptide-containing) forms of these β subunits were detected in the 20S and 26S fractions from *ump1*Δ cells (Figure 3). In addition, the proteasomes assembled in *ump1*Δ cells had reduced activity. Thus, Ump1p is required for the correct and efficient maturation of the proteasomes.

Partially Processed Forms of Pro-Pre2p Are Detected in the Proteasome Precursor Complex and the Proteasome of ump1Δ *Cells but Not in WT Cells*

These incompletely and prematurely processed forms of Pre2p (Figures 3A and 3B) appear to underlie a chymotrypsin-like activity that was associated with the precursor complex in *ump1*Δ cells but was absent from the same complex of wt cells (Figure 3C). This was suggested by the observation that this activity was not inhibited by treatment with lactacystin and was absent from cells lacking the Pre2p propeptide (P. C. R. and R. J. D., unpublished data). These findings indicated that Ump1p has a dual role in proteasome maturation. Specifically, Ump1p prevents premature processing of proPre2p in the precursor complex and is also required for the correct maturation of active sites upon assembly of the proteasome (see below).

Ump1p Is Degraded by the Newly Formed Proteasome

The kinetics of the rapid degradation of Ump1p is similar to the kinetics of processing of Pup1p (Figures 3B and 4A), suggesting that Ump1p is destroyed by the proteasome upon its formation from the two half-proteasome precursors and the accompanying maturation of active sites (Chen and Hochstrasser, 1996). This conclusion was strongly supported by the observation that Ump1p is significantly stabilized in the *pre1-1* mutant, which is deficient in the proteasome's chymotrypsin-like activity (Figure 4A). Specifically, in contrast to the pattern in wt cells, Ump1p was detectable in the 20S and 26S forms of the *pre1-1* proteasome. Ump1p in these complexes was shielded both from detection by antibodies and from digestion by trypsin (Figures 4B–4D). These and related data suggested a model in which Ump1p is encased within the 20S proteasome upon its assembly from the two Ump1p-containing half-proteasome precursor complexes (Figure 6). Formation of the 20S structure triggers active site maturation, resulting in the degradation of Ump1p.

The Propeptide of Pre2p Is Not Essential in ump1Δ *Cells*

Chen and Hochstrasser (1996) demonstrated that the propeptide of Pre2p is essential for cell viability and that this propeptide can operate in *trans*. They concluded that the propeptide, in addition to rendering proteasome precursors proteolytically inactive, serves a chaperone-like function required for the correct incorporation of Pre2p into the proteasome. One striking result of the present work is that the propeptide of Pre2p becomes dispensable in the *ump1*Δ mutant (Figure 5). In fact, the presence of the propeptide, which is essential for viability of wt cells, does not provide a growth advantage to *ump1*Δ cells that express it.

On the Functions of Ump1p and β Subunit Propeptides in Proteasome Maturation

Our results suggested a model illustrated in Figure 6. This model is based in part on the idea described by Chen and Hochstrasser (1996)—that the active sites of the proteasome are formed upon its assembly from two half-proteasome precursors, through a juxtaposition of subunits at the interface of the dyad-related halves. The advantage of this mechanism is that autocatalytic maturation of the active sites (through processing of the relevant β subunits) is coupled to the assembly of the proteasome, thus avoiding premature processing of the propeptides of β subunits. This view was supported by

Table 1. Yeast Strains

Strain	Relevant Genotype	Source/Comment
YPH500	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Sikorski and Hieter, 1989
JDC9-11	*MAT*α *ump1-1*	Derivative of YPH500
JD47-13C	*MAT***a** *his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52*	Dohmen et al., 1995
JD59	*MAT***a** *ump1-Δ1::HIS3*	Derivative of JD47-13C
JD53	*MAT*α	Derivative of JD47-13C
JD81-1A	*MAT*α *ump1-Δ1::HIS3*	Derivative of JD53
JD75	*MAT***a** *PRE1-ha::YIplac211 ump1-Δ1::HIS3*	Derivative of JD47-13C
JD126	*MAT***a** *UMP1-ha::YIplac128 PRE1-Flag-6His::YIplac211*	Derivative of JD47-13C
JD127	*MAT***a** *UMP1-ha::YIplac128 PRE1-ha::YIplac211*	Derivative of JD71
JD129	*MAT***a** *UMP1-ha::YIplac128*	Derivative of JD127
JD131	*MAT***a** *UMP1-ha::YIplac128 PUP1-ha::YIplac211*	Derivative of JD129
JD132	*MAT***a** *ump1-Δ1::HIS3 PUP1-ha::YIplac211*	Derivative of JD59
JD133	*MAT***a** *UMP1-ha::YIplac128 PRE2-ha::YIplac211*	Derivative of JD129
JD134	*MAT***a** *ump1-Δ1::HIS3 PRE2-ha::YIplac211*	Derivative of JD59
JD135	*MAT***a** *UMP1-ha::YIplac128 PRE3-ha::YIplac211*	Derivative of JD129
JD136	*MAT***a** *ump1-Δ1::HIS3 PRE3-ha::YIplac211*	Derivative of JD59
JD138	*MAT***a** *PRE2-ha::YIplac211*	Derivative of JD47-13C
JD139	*MAT***a** *PUP1-ha::YIplac211*	Derivative of JD47-13C
BBY45	*MAT***a** *his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52*	Bartel et al., 1990
JD61	*MAT***a**/*MAT*α *ump1-Δ1::HIS3/ump1-Δ2::LEU2*	Congenic with BBY45
JD50-10B	*MAT*α *leu2-3,112 his3-Δ200 trp1-1 ura3-52*	Congenic with BBY45
MHY952	*MAT*α *leu2-3,112 his3-Δ200 trp1-1 ura3-52* *pre2(=doa3)Δ1::HIS3* (YCpUbDOA3ΔLS-His) ($YEpDOA3_{LS}$)	Chen and Hochstrasser, 1996, congenic with BBY45
JD160	*MAT*α *ump1-Δ3* *pre2(=doa3)Δ1::HIS3* (YCpUbDOA3ΔLS-His) ($YEpDOA3_{LS}$)	Derivative of MHY952
JD163	*MAT*α *ump1-Δ3* *pre2(=doa3)-Δ1::HIS3* (YCpUbDOA3ΔLS-His)	Derivative of JD160, cured of $YEpDOA3_{LS}$
WCG4a	*MAT*α *ura3 his3-11 leu2-3,112*	Heinemeyer et al., 1993
YHI29/1	*MAT*α *pre1-1 ura3 his3-11 leu2-3,112*	Heinemeyer et al., 1993
JD150	*MAT*α *UMP1-ha::YIplac128*	Derivative of WCG4a
JD151	*MAT*α *pre1-1 UMP1-ha::YIplac128*	Derivative of YH129/1

biochemical analyses of the proteasome maturation in mammalian cells (Frentzel et al., 1994; Yang et al., 1995; Nandi et al., 1997; Schmidtke et al., 1997).

The results of the present work further support this model. In addition, we discovered that a novel factor, Ump1p, is required for autocatalytic active site maturation and proteasome assembly to occur in a coordinated fashion. A model that accounts for our findings and is consistent with the available evidence is illustrated in Figure 6. In this model, Ump1p has a chaperone-like function required for the efficient processing of the propeptides of β subunits upon the assembly of 20S proteasomes. Specifically, Ump1p interacts with these β subunits, most likely with their propeptides (structure A in Figure 6). Upon formation of the 20S proteasome (Figure 6, step 1), a conformational change of Ump1p, or a change of its position within the complex, would induce a conformational change of the propeptide that results in autocatalytic processing (Figure 6, step 2).

Why is the propeptide of Pre2p essential for proteasome assembly and activation *only* in the presence of Ump1p? We propose that the propeptide of Pre2p is required to induce an alteration of conformation or position of Ump1p upon the assembly of the 20S particle. In this model, the absence of the propeptide would leave Ump1p in a position that is incompatible with the formation of active proteasome. It is possible (and remains to be verified) that this chaperone-like function of a propeptide is unique to Pre2p, as it contains a much longer propeptide (75 residues) than the other β subunits.

In the model of Figure 6, Ump1p is a metabolically unstable chaperone that is required for the proper (and properly timed) processing of β subunits upon the assembly of the 20S proteasome and that is destroyed within the newly activated proteasome. The unusual mechanics of Ump1p, its noncatalytic mode of action, and its degradation by the protease it helps to activate characterize Ump1p as a novel type of molecular chaperone.

Experimental Procedures

Yeast Media

Yeast rich (YPD) and synthetic (S) minimal media with 2% dextrose (SD) or 2% galactose (SG) were prepared as described (Dohmen et al., 1995).

Isolation of the *UMP1* Gene

UMP1 was identified using a selection-based screen for mutants in the N-end rule pathway (reviewed by Varshavsky, 1996). *S. cerevisiae* strain YPH500 (Table 1) was transformed with two plasmids: pJD205, which expressed a Ura3p-based N-end rule reporter substrate (Arg-Tpi1p-Ura3p), and pRL2, which expressed Arg–β-gal, another N-end rule reporter substrate that can be monitored using X-Gal plate assays (Baker and Varshavsky, 1995). Previous work (Dohmen et al., 1994) has shown that rapid degradation of a Ura3-based N-end rule substrate renders the cells Ura^-, whereas mutants in the N-end rule pathway that express the same Ura3p-based reporter are Ura^+. In this screen, Ura^+ isolates were selected on SD media lacking uracil and were then tested on X-Gal plates to verify that the same isolates were also defective in the degradation of the Arg–β-gal N-end rule substrate. One of the mutants thus identified (*ump1-1*) was cured of pJD205 and transformed with a genomic yeast library (Rose et al., 1987). Six transformants yielded plasmids with four overlapping inserts that could restore the ability of cells to degrade Arg–β-gal. A 1981 bp BamHI/Sau3A fragment common

to all of the inserts was sequenced and found to contain one complete ORF and two flanking incomplete ORFs. Further mapping, using subcloning and PCR, confirmed that the complete ORF of 445 bp was responsible for the complementation. The *UMP1* sequence (EMBL database accession number: AJ002557) is identical to ORF YBR173C, an ORF subsequently identified by the yeast genome project.

Construction of Yeast Strains and Plasmids

Table 1 lists the strains used in this study. To construct *ump1*Δ alleles, the 1981 bp fragment described above was subcloned into M13mp19. Using single-stranded DNA of the resulting phage, a synthetic oligonucleotide and T4 DNA polymerase, the *UMP1* ORF was precisely deleted and replaced by a BglII restriction site. The resulting fragment was subcloned into pUC19, and the BglII site was used to insert fragments containing the *HIS3*, *LEU2*, or the *URA3* gene, the latter one being flanked by two direct repeats of a segment of the *E. coli hisG* gene (Alani et al., 1987). The resulting deletion alleles (*ump1-Δ1::HIS3, ump1-Δ2::LEU2*, and *ump1-Δ3::URA3*) were isolated as BamHI fragments, introduced into *S. cerevisiae*, and used to delete the *UMP1* gene (Rothstein, 1991). *ump1*Δ strains carrying an unmarked *ump1-Δ3* allele (resulting from recombination between the *hisG* repeats) were selected on plates containing 5-fluoroorotic acid (Alani et al., 1987). Construction of chromosomal ORFs that expressed C terminally tagged versions of Ump1p or proteasome subunits (Pre1p, Pre2p, Pre3p, and Pup1p) instead of their wt counterparts was performed as follows. Using primers that contained flanking EcoRI and KpnI sites, 3′ portions of the respective genes were amplified by PCR. These sites were then used to insert the amplified fragments into integrative plasmids based on YIplac128 (*LEU2* marked) or YIplac211 (*URA3* marked) (Gietz and Sugino, 1988) that contained sequences encoding epitope tags followed by the terminator sequence of the *CYC1* gene (T_{CYC1}). Each of the resulting plasmids was linearized within the coding sequence for targeted integration into the *S. cerevisiae* genome, yielding strains with one copy of the respective gene (fused in-frame to the tag-coding sequence) expressed from its natural promoter and T_{CYC1}, in addition to a 3′ portion of the same gene without promoter. The epitope tags used were double ha ("ha") and Flag-His_6 ("FH") (Dohmen et al., 1995). Plasmid pJD429 (*CEN/URA3*) expressing a *UMP1* cDNA from P*GAL1* was isolated from a cDNA library (Liu et al., 1992) through complementation of the *ump1*Δ mutation.

Pulse-Chase Analyses

R–β-gal (Ub-R–β-gal) and Ub-P–β-gal (Bachmair et al., 1986) were expressed in the *MAT***a** strains JD47-13C or JD59. MATα2_{1-68}–β-gal was expressed from the plasmid YCp50-α2_{deg1}–β-gal (Hochstrasser and Varshavsky, 1990) in the *MAT*α strains JD53 and JD81-1A (Table 1). Pulse labeling for 5 min with Redivue Promix [^{35}S] (Amersham) followed by a chase and immunoprecipitation with monoclonal anti–β-gal (Promega) or anti-ha (16B12, Babco) antibodies were carried out as described by Dohmen et al. (1991). ^{35}S proteins fractionated by SDS-PAGE were detected by fluorography.

Fractionation of Whole-Cell Extracts by Gel Filtration

S. cerevisiae were grown at 30°C in YPD or in SD media to OD_{600} of 1.4 ± 0.1, harvested at 3000g, washed with cold water, frozen in liquid nitrogen, and stored at −80°C. Cell paste was ground to powder in a mortar in the presence of liquid nitrogen. The extraction buffer was 50 mM Tris-HCl (pH 7.5), 2 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 15% glycerol, used at 2 ml per gram of pelleted yeast cells. After centrifugation at 31,000g for 10 min at 2°C, the supernatant was subjected to a second centrifugation at 60,000g for 30 min, yielding an extract with the protein concentration of ~5 mg/ml. The protein concentration of extracts was equalized in parallel experiments, using the extraction buffer. Using the FPLC system (Pharmacia), 200 μl samples of an extract were chromatographed on a Superose-6 column equilibrated with extraction buffer. The flow rate was 0.3 ml/min and fractions of 0.6 ml were collected. The Superose-6 column was calibrated using the following standards: thyroglobulin (669 kDa), ferritin (443 kDa), and bovine serum albumin (66 kDa). Dextran blue was used to monitor the void volume.

Assays for Proteolytic Activities with Fluorogenic Peptide Substrates

To determine the chymotrypsin-like activity, 20 μl of the protein fraction and 20 μl of 0.5 mM succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin in 50 mM Tris-HCl (pH 7.8), 2 mM ATP, 5 mM $MgCl_2$, and 1 mM DTT were mixed and incubated for 1 hr at 37°C. The reaction was stopped by addition of 960 μl of cold ethanol, and the fluorescence was measured at 440 nm, using the excitation wavelength of 380 nm. The trypsin-like activity and the peptidylglutamyl peptide-hydrolyzing activity were determined with, respectively, Cbz-Ala-Ala-Arg-4MeO-β-naphthylamide and Cbz-Leu-Leu-Glu-β-naphthylamide as fluorogenic peptide substrates (Fischer et al., 1994), except that the volume of the protein fraction was 75 μl, the reactions were stopped after 270 min at 37°C, and the fluorescence emission was measured at 366 nm, using an excitation wavelength of 420 nm. One unit (U) of a proteolytic activity is defined as 1 μmol of the fluorophore produced per min under these conditions.

Immunoprecipitation, Electrophoresis, and Immunoblotting

Immunoprecipitations were carried out using either monoclonal anti-tag antibodies (see below), or polyclonal rabbit antisera raised against the yeast 20S proteasome (a gift from K. Tanaka), or against Ump1p-His_6 expressed in *E. coli*. For immunoblotting, the protein samples were boiled for 3 min in the presence of 2% SDS and 0.1 M 2-mercaptoethanol, then subjected to 12% SDS-PAGE, and thereafter transferred onto a PVDF membrane (Millipore) in a wet blot system (BioRad). The blots were incubated with either rabbit anti-ubiquitin (Ramos et al., 1995), or anti-Cim3p (Ghislain et al., 1993) polyclonal antibodies, or 16B12 anti-ha (BabCo), or M2 anti-Flag (Kodak) monoclonal antibodies, and were processed as described (Ramos et al., 1995), except that the initially blotted proteins were visualized using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgGs, the chemiluminescence blotting substrate detection system from Boehringer Mannheim, and X-ray films. Nondenaturing 4.5% acrylamide gel electrophoresis was performed as described by Hough et al. (1987), and the gels were incubated for 15 min in transfer buffer containing 0.1% SDS before electrotransfer.

Affinity Purification of Proteasomes and Related Complexes

The 20S proteasome and its precursors were purified from strain JD126 that expressed Pre1p-FH and Ump1p-ha. Crude extracts were prepared as described above, followed by an exchange of buffer to 50 mM Na-phosphate (pH 8.0), 0.3 M NaCl, using PD-10 columns (Pharmacia). The extract was then incubated with Ni-NTA agarose (Qiagen) in batch for 2 hr at 4°C, followed by washings and elution according to the manufacturer's protocol, except that the (pH 6.0) washing buffer contained 20 mM imidazole. His_6-tagged proteins were eluted with a step gradient of 100-500 mM imidazole. Active fractions containing a mixture of the mature proteasome and its precursors were pooled, diluted 2-fold with 50 mM Tris-HCl (pH 7.5), and incubated for 2 hr at 4°C in batch with 0.5 ml anti-Flag M2 antibody affinity resin (IBI/Eastman Kodak) that had been equilibrated in TN buffer (0.15 M NaCl, 50 mM Tris-HCl, [pH 7.5]). After the loading, the resin was washed with TN buffer. Flag-tagged proteins were specifically eluted with the Flag epitope peptide (IBI/Eastman Kodak).

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