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Eukaryotic 20S proteasome catalytic subunit propeptides prevent active site inactivation by N-terminal acetylation and promote particle assembly

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Proteins targeted for degradation by the ubiquitin–proteasome system are degraded by the 26S proteasome. The core of this large protease is the 20S proteasome, a barrel-shaped structure made of a stack of four heptameric rings. Of the 14 different subunits that make up the yeast 20S proteasome, three have proteolytic active sites: Doa3/β5, Pup1/β2 and Pre3/β1. Each of these subunits is synthesized with an N-terminal propeptide that is autocatalytically cleaved during particle assembly. We show here that the propeptides have both common and distinct functions in proteasome biogenesis. Unlike the Doa3 propeptide, which is crucial for proteasome assembly, the Pre3 and Pup1 propeptides are dispensable for cell viability and proteasome formation. However, mutants lacking these propeptide-encoding elements are defective for specific peptidase activities, are more sensitive to environmental stresses and have subtle defects in proteasome assembly. Unexpectedly, a critical function of the propeptide is the protection of the N-terminal catalytic threonine residue against N^{α}-acetylation. For all three propeptide-deleted subunits, activity of the affected catalytic center is fully restored when the Nat1-Ard1 N^{α}-acetyltransferase is mutated. In addition to delineating a novel function for proteasome propeptides, these data provide the first biochemical evidence for the postulated participation of the α-amino group in the proteasome catalytic mechanism.

Keywords: N^{α} acetylation/assembly/NAT1 gene/20S proteasome/ubiquitin

Introduction

The ubiquitin–proteasome system is the major mechanism for regulated degradation of intracellular proteins in eukaryotes. Short-lived proteins such as cell cycle regulators and transcription factors, as well as abnormal proteins from the cytosol, nucleus and endoplasmic reticulum, are specifically recognized and polyubiquitinated by enzymes in the ubiquitin-conjugation pathway (Coux *et al*., 1996; Hochstrasser, 1996; Hershko and Ciechanover, 1998). Modified substrates are degraded by the 26S proteasome, which is made up of two 19S regulatory complexes and a proteolytically active 20S proteasome core (Rubin and Finley, 1995; Coux *et al*., 1996; Hochstrasser, 1996; Baumeister *et al*., 1998).

20S proteasomes with related quaternary structures are found in all three branches of life (Baumeister *et al*., 1998). Subunits are arranged in a stack of four heptameric rings, forming a barrel-shaped structure with a central channel. The outermost rings are composed of α-subunits and the inner ones of β-subunits. The β-subunits house the proteolytic active sites on the interior of the complex (Löwe *et al*., 1995; Seemüller *et al*., 1995), and substrate is believed to enter through narrow pores in the α rings. While the proteasome of the archaeon *Thermoplasma acidophilum* is composed of homomeric α and β rings (Zwickl *et al*., 1992; Löwe *et al*., 1995), in *Saccharomyces cerevisiae* and other eukaryotes there are seven unique subunits within each α and each β ring (Heinemeyer *et al*., 1991; Chen and Hochstrasser, 1995; Groll *et al*., 1997). Of the seven β-subunits in yeast, only three bear protease active sites: Doa3/β5, Pup1/β2 and Pre3/β1. The yeast proteasome has been characterized as having chymotrypsin-like, trypsin-like and postglutamyl peptide hydrolyzing (PGPH) activities toward small peptide substrates. We and others have recently demonstrated by mutational analysis that the Doa3, Pup1 and Pre3 subunits, respectively, are responsible for these peptidase activities (Chen and Hochstrasser, 1996; Arendt and Hochstrasser, 1997; Heinemeyer *et al*., 1997). The Doa3 active site is by far the most critical of the three for cell growth and degradation of ubiquitinated substrates (Arendt and Hochstrasser, 1997).

Insight into the catalytic mechanism of β-subunits was gained from crystal structures of the *Thermoplasma* and yeast 20S proteasomes (Löwe *et al*., 1995; Groll *et al*., 1997). These structures implicated the hydroxyl group of the N-terminal threonine residue as the catalytic nucleophile, an inference supported by mutagenesis (Seemüller *et al*., 1995; Chen and Hochstrasser, 1996). The proteolytic mechanism of proteasomes was suggested to be similar to that of serine proteases, which also make use of a hydroxyl nucleophile. As a necessary first step in catalysis in the serine proteases, the serine hydroxyl donates its proton to a nearby basic group (Perona and Craik, 1995). In the case of the proteasome, the proton acceptor could be a highly conserved lysine, Lys33, or the α-amino group, both of which are found near the Thr1 hydroxyl. The yeast and *Thermoplasma* structures suggest that the α-amino group, which is expected to have a more favorable pK_a than Lys33, is likely to be the proton acceptor, although Lys33 is essential for catalytic activity (Chen and Hochstrasser, 1996; Schmidtke *et al*., 1996; Seemüller *et al*., 1996; Heinemeyer *et al*., 1997).

All active β-subunits are synthesized with an N-terminal propeptide, which is autoprocessed at the end of proteasome assembly to reveal the catalytic Thr1 residue (Chen and Hochstrasser, 1996; Schmidtke *et al*., 1996; Seemüller *et al*., 1996). Many other proteases are expressed with N-terminal propeptides that serve as inhibitors or

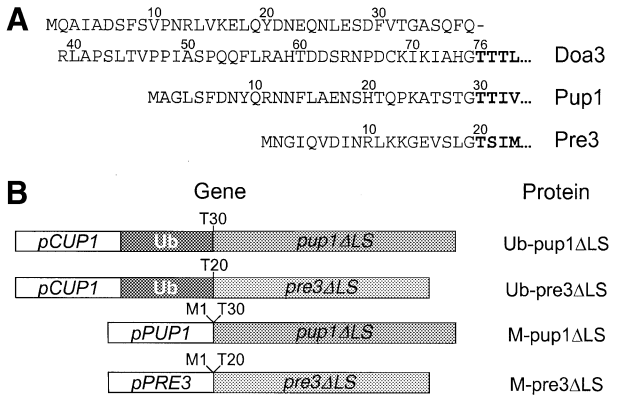

Fig. 1. Propeptides of the yeast proteasome active β-subunits. (**A**) Propeptide sequences of the Doa3, Pup1 and Pre3 subunits. Amino acids downstream of the propeptide processing site are shown in bold. (**B**) Expression constructs for mutant subunits.

folding catalysts (Khan and James, 1998), and the propeptides of proteasome β-subunits could play similar roles. They may act in proteasome assembly as well. Assembly of eukaryotic and eubacterial proteasomes appears to take place via a half-proteasome intermediate which contains one α ring and a ring of β-subunits with unprocessed β-subunit precursors (Yang *et al*., 1995; Nandi *et al*., 1997; Schmidtke *et al*., 1997; Zühl *et al*., 1997). Propeptide processing occurs following the apposition of two such intermediates, yielding a mature proteasome particle (Chen and Hochstrasser, 1996). The propeptides of the eubacterial *Rhodococcus* β-subunits appear to promote subunit folding and probably also act at a late step of proteasome assembly (Zühl *et al*., 1997). The yeast Doa3/β5-subunit and its mammalian homolog Lmp7 cannot incorporate into proteasomes *in vivo* without their propeptides (Cerundulo *et al*., 1995; Chen and Hochstrasser, 1996). However, the mechanisms of action of these propeptides in proteasome biogenesis have not been defined, and it is not known whether divergent propeptides have common or distinct functions.

In the present work we show that, unlike the Doa3 propeptide, the propeptides of Pup1 and Pre3 are dispensable for proteasome assembly *in vivo*, but their deletion leads to subtle defects in the assembly process. We also demonstrate that Doa3, Pup1 and Pre3 propeptide-deletion mutants become targets of a cellular N^{α}-acetyltransferase. All three mutants are at least partially inactivated by N-terminal acetylation, suggesting a general role for β-subunit propeptides in protecting the α-amino group from modification. The data also demonstrate a biochemical requirement for a free α-amino group in catalysis.

Results

Pup1 and Pre3 propeptides are dispensable for cell growth

The propeptides of the three active 20S proteasome β-subunits in yeast vary from 75 to 19 amino acids in length (Figure 1A) and are not closely related in sequence. Near the completion of proteasome assembly, each subunit is processed at a conserved Gly-Thr motif to expose the catalytic N-terminal Thr residue (Chen and Hochstrasser, 1995, 1996). The 75-residue Doa3 propeptide is essential for cell growth and proteasome assembly and is able to function *in trans*, i.e. when expressed separately from the rest of the subunit. Nothing was known about the functions of the Pup1 and Pre3 propeptides. To study the roles of these propeptides, we prepared propeptide-deletion constructs in two ways (Figure 1B). In the first, the propeptide-encoding regions of *PUP1* and *PRE3* were replaced with the ubiquitin coding sequence. When expressed in yeast, ubiquitin is rapidly processed from such linear protein fusions by cellular deubiquitinating enzymes. These protein fusions are referred to as Ub-pup1ΔLS and Ub-pre3ΔLS (LS, leader sequence), and the alleles were expressed from the yeast *CUP1* promoter. In the second set of constructs, propeptide-encoding sequences were simply deleted. These constructs place an initiator Met codon directly before the catalytic Thr codon and retain the cognate upstream DNA regulatory sequences (*M-pup1ΔLS* and *M-pre3ΔLS*). The N-terminal Met-Thr dipeptide of the resulting fusion is expected to be a good substrate for yeast methionine aminopeptidase (Bradshaw *et al*., 1998), so these constructs should also produce a free Thr at the N-terminus. 'Ub-' and 'M-' constructs gave similar results in all experiments.

In contrast to the lethality of the *Ub-doa3ΔLS* mutation (Chen and Hochstrasser, 1996), *pup1* and *pre3* propeptide-deletion mutants showed only slight growth defects under a variety of conditions used to detect defects in the ubiquitin–proteasome system. The *pup1ΔLS* mutants were slightly hypersensitive to 1 μg/ml canavanine, strongly temperature sensitive and hyperresistant to 30 μM cadmium (Figure 2; data not shown). Under all conditions assayed, the *pup1ΔLS* mutation was more deleterious than the *pup1-T30A* allele, whose product is completely defective for trypsin-like peptidase activity and propeptide processing (Arendt and Hochstrasser, 1997). The *pre3ΔLS* mutants had at most mild growth defects at 30 and 37°C and on canavanine compared with *PRE3* cells, while the *pre3-T20A* mutant grew exactly like wild type under these conditions. Like the *pup1ΔLS* strains, the *pre3ΔLS* mutants were also cadmium hyperresistant, which we had shown previously correlates with very weakly compromised proteasome activity (Arendt and Hochstrasser, 1997). In both cases, then, the phenotypic effects of the propeptide-deletion mutations were modest but slightly stronger than those of the corresponding Thr to Ala mutations.

Functional defects of propeptide-deletion mutants

The propeptide-deletion strains were also tested for their ability to degrade *in vivo* substrates of the ubiquitin–proteasome system (Figure 3A). The MATα2 repressor has a half-life of ~5 min in wild-type cells. In *pup1ΔLS* cells, this half-life was increased ~1.8-fold. An engineered substrate, Leu-β-galactosidase (Leu-βgal) (Bachmair *et al*., 1986), was strongly stabilized in *pup1ΔLS* cells. However, *pre3ΔLS* mutants showed no measurable defect in the *in vivo* degradation of either substrate. These weak, but reproducible, effects on *in vivo* proteolysis correlated with the limited growth defects of the *pup1ΔLS* and *pre3ΔLS* mutant strains (Figure 2).

The proteasome is essential for viability in yeast, and so any gross defects in its assembly would be lethal. That the *pup1ΔLS* and *pre3ΔLS* mutants have only slight growth defects indicates that sufficient numbers of proteasomes

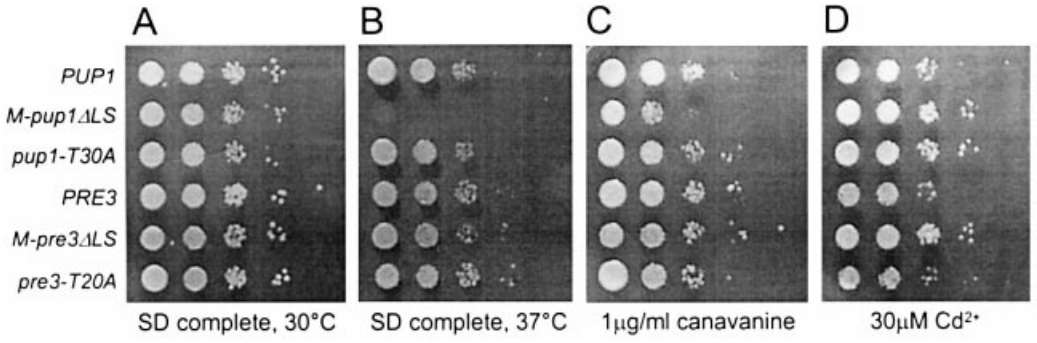

Fig. 2. Effects on growth of Pup1 and Pre3 propeptide deletions. (**A**) Growth of cells lacking the coding sequences for the Pup1 or Pre3 propeptides. Ten-fold serial dilutions of logarithmic cultures were spotted on minimal medium and incubated at 30°C for 2 days. From top to bottom, strains were MHY1217, MHY1505, MHY1376, MHY1156, MHY1508 and MHY1157. (**B**) As in (A) except that cells were placed at 37°C for 3 days. (**C**) As in (A) except that cells were grown on minimal medium containing 1.0 μg/ml canavanine sulfate for 3 days at 30°C. (**D**) As in (A) except that cells were grown on minimal medium containing 30 μM $CdCl_2$ for 3 days at 30°C.

are assembled in the mutant cells to support near-normal cell function. Consistent with this, epitope-tagged derivatives of these propeptide-deleted subunits were found almost exclusively in the 20S proteasome-containing fractions of glycerol gradient-fractionated extracts (Figure 3B) (the pup1ΔLS-HA_2 protein runs as a doublet; the basis of this is described below). It was still possible, however, that the rate and/or efficiency of assembly might be decreased. To measure the rate of proteasome assembly in the *pup1ΔLS* and *pre3ΔLS* strains, we assayed the rate of proDoa3 processing. Chymotrypsin-like activity was at wild-type levels in both *pup1ΔLS* and *pre3ΔLS* cells, so we could infer that Doa3 was processed normally in these mutants. However, if the Pup1 and Pre3 propeptide-deletion mutants slowed proteasome assembly at a step(s) before or during Doa3 processing, this slow-down should be reflected by a drop in the observed rate of Doa3 processing. Cleavage of the Doa3 propeptide is one of the final steps in proteasome assembly (Chen and Hochstrasser, 1996).

Strains were constructed that expressed His_6-tagged Doa3 together with wild-type Pup1 or Pre3 or the corresponding propeptide-deleted derivative. ProDoa3-His_6 processing was followed by pulse–chase analysis (Figure 3C), and the rate of processing was defined as the disappearance of full-length proDoa3. Processing was 2.5-fold slower in *Ub-pup1ΔLS* cells than in the congenic wild-type strain, and 1.5-fold slower in *Ub-pre3ΔLS* cells. The band marked with an arrow appears to be a naturally occurring Doa3 processing intermediate, since it was present during the first time point in both wild-type strains. However, it persisted in the *Ub-pup1ΔLS* strain. In addition, some aberrant Doa3 products that ran more quickly than the properly processed protein ($Doa3_m$) were detected in *Ub-pre3ΔLS* cells (Figure 3C, asterisks). When extracts from these *Ub-pre3ΔLS DOA3-*His_6 cells were separated on glycerol gradients, the aberrant Doa3 species were only detected in complexes smaller than 20S proteasomes (data not shown). They are therefore likely to represent Doa3 subunits trapped and proteolyzed in dead-end assembly products. We conclude that the *pup1ΔLS* mutant primarily affects the rate of proteasome assembly, while the *pre3ΔLS* mutant also affects its fidelity.

Propeptide-deletion mutants suffer loss of peptidase activity

Since the propeptide-deletion constructs were made in such a way as to produce a subunit identical to the one that exists within an active proteasome, i.e. with all catalytic residues intact and propeptide removed, we did not expect the mutant subunits to display specific peptidase defects. Each of the three β-subunit active sites has a distinct specificity for small fluorogenic peptide substrates, allowing the activity of each active site to be assayed in preparations of glycerol gradient-purified 20S proteasomes. Surprisingly, when pup1ΔLS and pre3ΔLS proteasomes were analyzed in this way, each mutated subunit displayed a striking drop in its corresponding peptidase activity (Figure 4). While chymotrypsin-like activity was not significantly reduced in either case, the *pre3ΔLS* mutants showed a complete loss of PGPH activity and a small reduction in trypsin-like activity. Similarly, the *pup1ΔLS* mutants suffered a 60% loss of trypsin-like activity and a minor loss of PGPH activity.

The pre3ΔLS subunit is N-terminally acetylated in vivo

The observation that propeptide-deleted subunits display strong defects in peptidase activity was unexpected. We selected the *pre3ΔLS* mutant to investigate this phenomenon further. One explanation for the total loss of PGPH activity in this strain might be that when the N-terminal threonine is prematurely exposed to the cytosol, it is cleaved by an aminopeptidase. To test this possibility, we purified 20S proteasomes from *Ub-pre3ΔLS* cells, separated the subunits by two-dimensional gel electrophoresis, and following blotting to PVDF, subjected the pre3ΔLS spot to N-terminal sequencing. No sequence was obtained in two attempts, despite the presence of ample protein, strongly suggesting that the N-terminus was blocked.

The most common N^{α}-amino modification in eukaryotes is acetylation (Bradshaw *et al*., 1998). In *S.cerevisiae*, proteins with small penultimate residues (e.g. Thr) are cotranslationally processed by methionine aminopeptidase and then acetylated by the N^{α}-acetyltransferase Nat1-Ard1. Deletion of either the *NAT1* or *ARD1* gene is sufficient to abolish this activity (Mullen *et al*., 1989). We

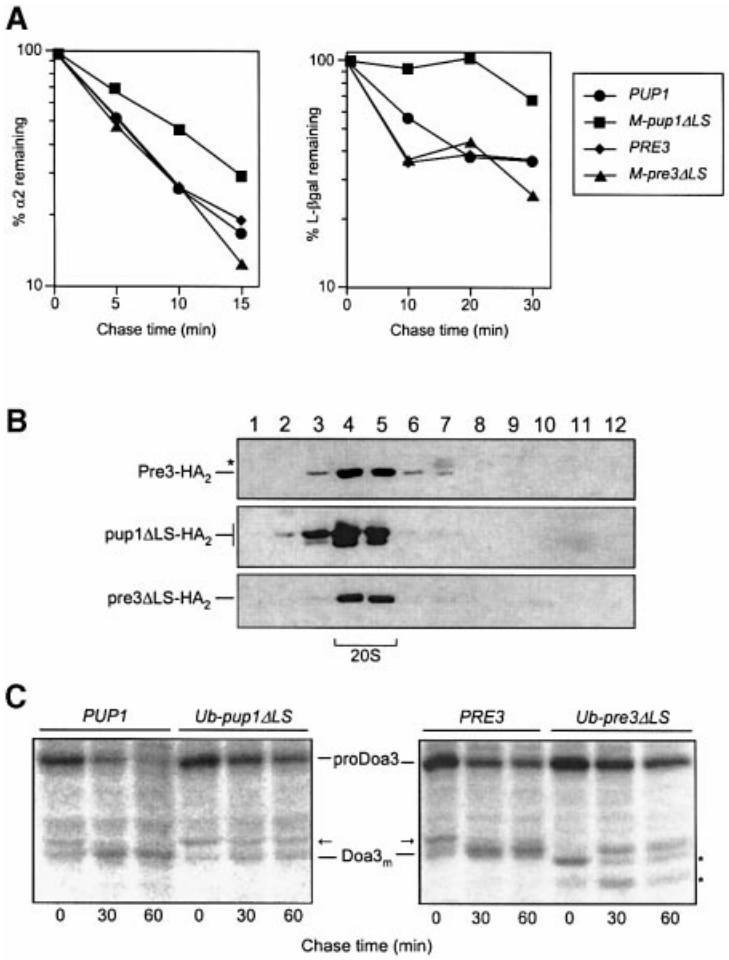

Fig. 3. Proteasomal defects in mutants lacking Pre3 or Pup1 propeptide sequences. (**A**) Pulse–chase analysis of MATα2 and Leu-βgal degradation in *PUP1, M-pup1ΔLS, PRE3* and *M-pre3ΔLS* cells. Left: MATα2 degradation at 30°C. The half-life of α2 was ~5 min in both wild-type strains (MHY1506 and MHY1413) and in *M-pre3ΔLS* cells (MHY1508), and was ~9 min in *M-pup1ΔLS* cells (MHY1507). Right: Degradation of Leu-βgal at 30°C. Strains were MHY1503, MHY1505, MHY1413 and MHY1508. (**B**) Mutant subunit incorporation into 20S proteasomes. Extracts were separated on 10–40% glycerol gradients, and each fraction was probed by anti-HA immunoblotting. Strains are MHY1413, MHY1508 and MHY1505, from top to bottom. 20S proteasome activity peaked in fractions 4–5 in each gradient. The asterisk in the uppermost panel indicates a species in fraction 7 that is likely to be the unprocessed form of Pre3-HA_2 in what may be an assembly intermediate. (**C**) Doa3 processing defects in pre3ΔLS and pup1ΔLS cells. MHY1513, MHY1514, MHY1515 and MHY1516 were pulse-labeled for 15 min, and chased for the indicated times. Samples were processed as described in Materials and methods, and immunoprecipitated Doa3-His_6 was detected by fluorography. Positions of unprocessed (proDoa3) and processed ($Doa3_m$) subunit are indicated. The arrow indicates a possible processing intermediate. The asterisks indicate two aberrant Doa3 products produced in the *pre3ΔLS* strain.

constructed a strain that combined a deletion of the *NAT1* gene with the *Ub-pre3ΔLS* mutation and then purified 20S proteasomes from the double mutant. When proteasome subunits were separated by two-dimensional gel electrophoresis, the pre3ΔLS spot was shifted toward the basic end of the gel relative to its position in *Ub-pre3ΔLS* proteasomes (Figure 5, spot 13). This shift is consistent with restoration of a positive charge at the Pre3 N-terminus. That a single pre3ΔLS spot was seen in proteasomes from both strains suggests that most or all pre3ΔLS subunits in *Ub-pre3ΔLS* proteasomes were blocked, and that most or all of these subunits were deblocked in *nat1*Δ cells. [Several other spots, most of them α-subunits either known or predicted to be N-terminally blocked, also shifted position in the *nat1*Δ background (Figure 5B, open arrowheads).] N-terminal sequencing of the pre3ΔLS spot isolated from *nat1*Δ *Ub-pre3ΔLS* proteasomes revealed the predicted N-terminal sequence, TSIMAVT, confirming that the previous block to pre3ΔLS sequencing was due to N^{α}-acetylation of the subunit. These data provide direct evidence that in the absence of its propeptide, Pre3 retains the catalytic Thr residue but is subject to N^{α}-acetylation by Nat1-Ard1.

We then asked if this acetylation event could account for the defects in peptidase activity of *pre3ΔLS* proteasomes. Remarkably, deletion of *NAT1* in the *pre3ΔLS* strain led to a complete restoration of PGPH activity (Figure 6A, left panel). Similar results were also obtained with *ard1*Δ *pre3ΔLS* cells (data not shown). We conclude that acetylation of the α-amino group of pre3ΔLS by Nat1-Ard1 abolishes activity of the Pre3 active site, implicating the α-amino group in the catalytic mechanism of this β-subunit.

pup1ΔLS and doa3ΔLS are also inactivated in a Nat1-dependent manner

We had observed a significant defect in trypsin-like activity of the *pup1ΔLS* mutants (Figure 4) and reasoned that this

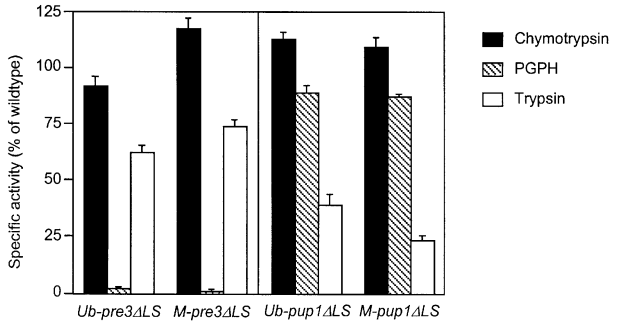

Fig. 4. Peptidase activities of *pre3ΔLS* and *pup1ΔLS* mutant strains. Extracts of mutant and congenic wild-type strains were prepared and separated on 10–40% glycerol gradients. Proteasome-containing fractions were pooled and assayed for each peptidase activity. Proteasome levels were measured by quantitative [^{125}I]protein A Western blotting using anti-20S polyclonal antibodies; these values were used to calculate relative specific activities. Error bars indicate the standard deviation of triplicate measurements. Pairs of mutant and wild-type strains, from left to right, were MHY1216 and MHY1217, MHY1505 and MHY1503, MHY1377 and MHY1156, and MHY1508 and MHY1413.

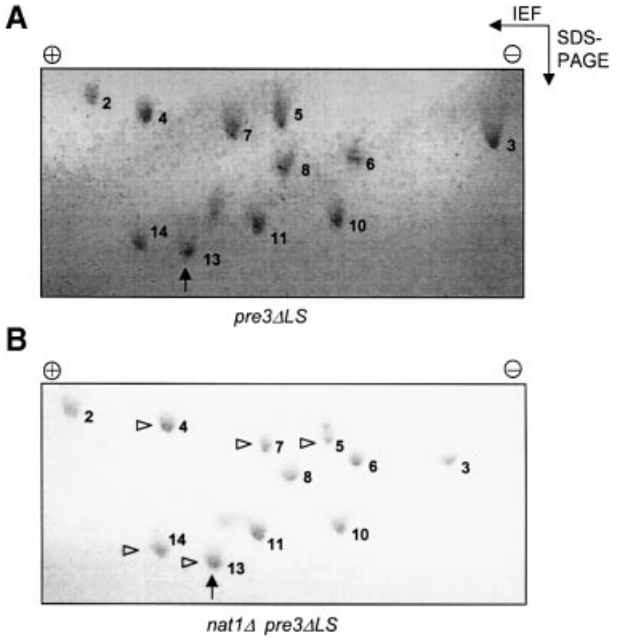

Fig. 5. Two-dimensional gel analysis of purified 20S proteasomes from *Ub-pre3ΔLS* and *nat1Δ Ub-pre3ΔLS* mutants. (**A**) Subunits of pre3ΔLS proteasomes (MHY1377) separated by two-dimensional gel electrophoresis, transferred to membrane and stained with Coomassie Blue. Pre3 (subunit 13) is indicated by the arrow. Proteins were separated in the horizontal dimension by isoelectric focusing (position of anode and cathode are indicated), and by 12.5% SDS–PAGE in the vertical dimension. Subunit numbering is taken from Heinemeyer *et al.* (1991). (**B**) 20S proteasome subunits from *nat1Δ Ub-pre3ΔLS* cells (MHY1373) analyzed as in (A). Open arrowheads indicate subunit spots that are shifted to the basic end of the isoelectric focusing gel compared with the *NAT1* strain. Pre3 is indicated with an arrow. Spots 10 (Doa3) and 11 (Pre1) do not shift and serve as fiducials.

might be due to N^{α}-acetylation of this subunit. As shown in Figure 6A (middle panel), inactivation of *NAT1* restored wild-type levels of trypsin-like activity to the *Ub-pup1ΔLS* strain, suggesting that pup1ΔLS is N^{α}-acetylated and inactivated in a *NAT1*-dependent manner. Pup1ΔLS from *NAT1* cells also migrated anomalously on SDS–PAGE

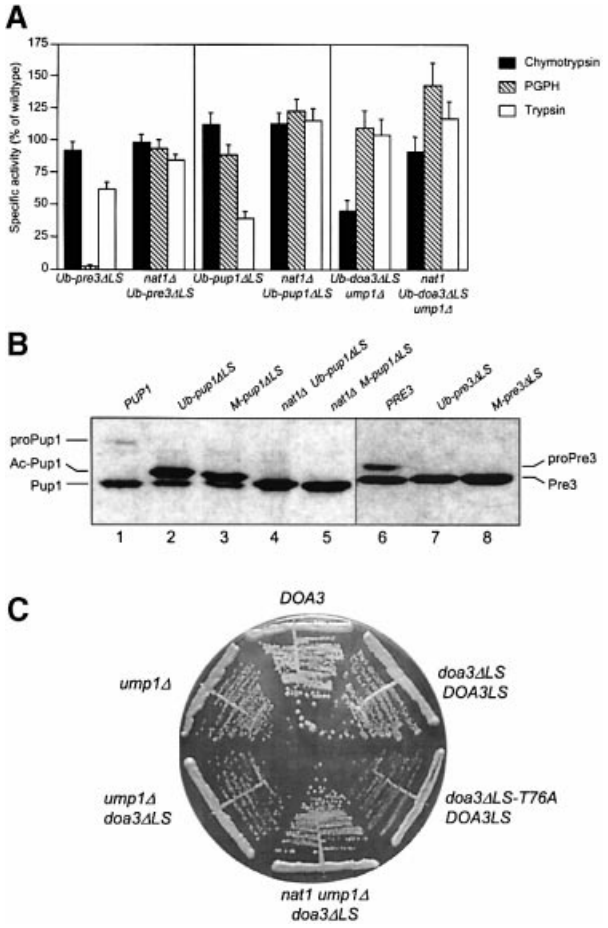

Fig. 6. Disruption of the N^{α}-acetyltransferase gene *NAT1* restores activity to proteasomes with propeptide-deleted β-subunits. (**A**) Normalized peptidase activities of *nat1Δ Ub-pre3ΔLS*, *nat1Δ Ub-pup1ΔLS* and *nat1 ump1Δ Ub-doa3ΔLS* compared with congenic *NAT1* strains. 20S proteasomes were assayed for activity as described for Figure 4. *NAT1* and *nat1* mutant strain pairs were, from left to right: MHY1156 and MHY1377 (from Figure 4), MHY1372 and MHY1373, MHY1217 and MHY1216 (from Figure 4), MHY1374 and MHY1375, MHY803 and MHY1327, MHY803 and MHY1471. (**B**) Anti-HA immunoblot analysis. Extracts from the strains indicated were prepared from logarithmically growing cells by lysis in SDS loading buffer, separated on 12.5% SDS gels and immunoblotted with anti-HA antibodies. Strains were MHY1503 (lane 1), MHY1504 (lane 2), MHY1505 (lane 3), MHY1510 (lane 4), MHY1511 (lane 5), MHY1413 (lane 6), MHY1414 (lane 7) and MHY1508 (lane 8). (**C**) Growth of Doa3 propeptide-deletion mutants. Strains were streaked on YPD and incubated at 30°C for 2 days. Clockwise from the top: MHY803, MHY952, MHY973, MHY1471, MHY1327, MHY1326.

gels. The subunit ran as two discrete bands (Figure 6B, lanes 2 and 3): one which comigrated with the correctly processed species (lane 1) and one which migrated more slowly. Pulse–chase experiments indicated that the two bands were present in a constant ratio (~60% upper, ~40% lower) during a 60 min chase (data not shown), suggesting that the more slowly migrating band was produced as rapidly as the lower one. The presence of the upper band was also *NAT1* dependent, as it disappeared when *NAT1* was disrupted (lanes 4 and 5). The fraction of Pup1 in the slow-migrating species coincided with the 60% loss of trypsin-like activity in pup1ΔLS proteasomes, and presumably represented the fraction of pup1ΔLS that is N^{α}-acetylated (see Figure 6A). Though the aberrantly

migrating band was produced in a *NAT1*-dependent manner, we do not know if such a difference in migration was caused by N^{α}-acetylation alone. The pre3ΔLS polypeptide, which was fully acetylated, comigrated with the processed wild-type subunit (Figure 6B, lanes 6–8).

Finally, we asked if the Doa3 subunit was also subject to N^{α}-acetylation when its propeptide was missing. This experiment was complicated by the fact that the *Ub-doa3ΔLS* allele is normally lethal. However, when combined with a deletion of *UMP1*, which encodes a proteasome assembly factor, *doa3ΔLS* cells are able to grow, albeit poorly (Ramos *et al*., 1998). Proteasome activities were therefore measured in *ump1Δ Ub-doa3ΔLS* and *nat1 ump1Δ Ub-doa3ΔLS* strains. As with pre3ΔLS and pup1ΔLS, we observed a specific, *NAT1*-dependent drop in the peptidase activity associated with the mutated subunit (chymotrypsin-like; Figure 6A, right panel). This result suggests that doa3ΔLS is also N^{α}-acetylated, probably to ~50% of the population. Expression of the Doa3 propeptide (Doa3LS) from a separate plasmid can also rescue the lethality of the *Ub-doa3ΔLS* mutant (Chen and Hochstrasser, 1996). *Ub-doa3ΔLS* cells with the *trans*-expressed propeptide still had only ~50% of the chymotrypsin-like activity seen with the wild-type *DOA3* control (data not shown), demonstrating that the propeptide cannot protect the subunit from inactivation when it is expressed *in trans*.

The catalytic activity of the Doa3 subunit is central to *in vivo* protein degradation and cell growth (Chen and Hochstrasser, 1996; Arendt and Hochstrasser, 1997) (Figure 6C, *doa3ΔLS-T76A DOA3LS*). Consistent with this, we observed an increase in growth rate of the *nat1 ump1Δ Ub-doa3ΔLS* mutant, which had wild-type levels of chymotrypsin-like activity, versus the *ump1Δ Ub-doa3ΔLS* mutant, which retained only 50% of this activity (Figure 6C). We also note that although the *Ub-doa3ΔLS DOA3LS* mutant was as defective for chymotrypsin-like activity as the *ump1Δ Ub-doa3ΔLS* mutant, its growth impairment was much less severe. This result suggests that additional defects exist in the *ump1Δ Ub-doa3ΔLS* mutant. These deficiencies are likely to be in proteasome assembly, as Ump1 is a proteasome assembly factor (Ramos *et al*., 1998), and ~50% fewer proteasomes were detected in this strain compared with the wild-type *DOA3* control after glycerol-gradient fractionation of equal amounts of total protein (data not shown).

The pre3ΔLS pup1ΔLS double mutant displays enhanced phenotypic defects

To determine whether there was a genetic interaction between the *pup1* and *pre3* propeptide deletions, we constructed a *pre3ΔLS pup1ΔLS* double mutant. The double mutant grew significantly more slowly than either single mutant (Figure 7A), and showed enhanced defects in α2 repressor degradation (data not shown). As with each of the single mutants, chymotrypsin-like activity was not decreased, but rather was somewhat activated, and PGPH-like activity was undetectable, as in the *pre3ΔLS* mutant (Figure 7B). The drop in trypsin-like activity in the double mutant corresponded to a roughly additive defect relative to each single mutant: a loss of ~60% of activity that appears to be due to N^{α}-acetylation of the pup1ΔLS subunit, compounded by a ~40% drop in the remaining activity, resulting from the *Ub-pre3ΔLS* mutation (compare with Figure 4, left panel), leaving only ~24% of trypsin-like activity relative to wild type. Pup1 and Pre3 are direct neighbors within each β ring, but it is not yet clear how deletion of the Pre3 propeptide leads to a defect in the activity of the Pup1 subunit.

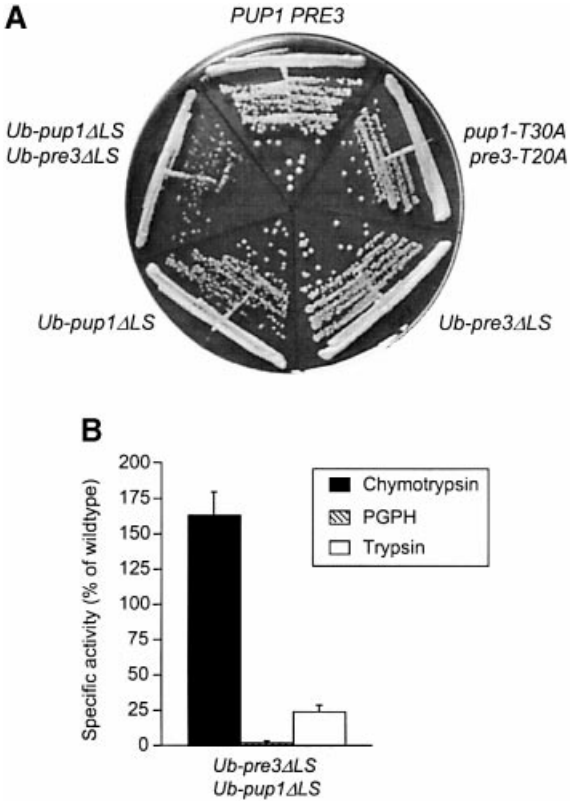

Fig. 7. Defects of the *pre3ΔLS pup1ΔLS* double mutant. (**A**) Cells were streaked on YPD medium and incubated at 30°C for 2 days. Doubling times for each strain were calculated from log phase cultures grown in YPD at 30°C. Clockwise from the top (doubling time in hours): MHY1177 (1.8), MHY1178 (2.0), MHY1377 (1.9), MHY1216 (2.0) and MHY1493 (2.5). (**B**) Normalized peptidase activities of the *Ub-pup1ΔLS Ub-pre3ΔLS* mutant (MHY1493) compared with the congenic wild-type strain (MHY1177). 20S proteasomes were assayed for activity as in Figure 4.

The double propeptide-deletion mutant and the *pup1-T30A pre3-T20A* mutant have nearly identical defects in PGPH- and trypsin-like activities, yet the latter strain grows much better (Figure 7). The additional growth defect of the *Ub-pre3ΔLS Ub-pup1ΔLS* mutant could be at least partially attributed to its 20% lower 20S proteasome levels at steady-state relative to wild type (data not shown). Simultaneous absence of the two propeptides during maturation may result in a structural perturbation that affects the ability of the mutant proteasomes to act on protein substrates. Alternatively, inactive propeptide-deleted subunits may be involved in unproductive substrate interactions that slow proteolysis, while the corresponding Thr to Ala mutants retain their propeptides, which could limit substrate binding.

Discussion

We have demonstrated here that the three active β-subunit propeptides make strikingly different contributions to

Table I. *pre3* and *pup1* strains

	YCplac22-P_[a]	YCplac22-P_-HA_2	YCplac22-Ub-p_ΔLS	YCplac22-Ub-p_ΔLS-HA_2	YCplac22-M-p_ΔLS-HA_2
*pre3*Δ[b]	MHY1156	MHY1413	MHY1377	MHY1414	MHY1508
*nat1*Δ *pre3*Δ	MHY1372	–	MHY1373	–	–
*pup1*Δ	MHY1217	MHY1503	MHY1216	MHY1504	MHY1505
*nat1*Δ *pup1*Δ	MHY1374	–	MHY1375	MHY1510	MHY1511

[a]P_ indicates either PRE3 or PUP1, as appropriate to cover the chromosomal deletion indicated in the left column. *URA3*-marked plasmids carrying the wild-type alleles in each deletion strain were evicted on 5-FOA plates.
[b]Deletion strains are MHY1031 (*pre3*Δ), MHY1331 (*nat1*Δ *pre3*Δ), MHY1026 (*pup1*Δ) and MHY1355 (*nat1*Δ *pup1*Δ).

Table II. Yeast strains not listed in Table I

Strain	Genotype
MHY500[a]	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1*
MHY501[a]	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1*
MHY691[b]	*MAT***a** *his3-11 leu2-3,112 ura3-1 trp1-1 ade2-1 can1-100 nat1-5::LEU2*
MHY1331	*MATα his3 leu2-3,112 ura3 trp1-1 can1-100 nat1-5::LEU2 pre3-Δ2::HIS3* [pRS316-PRE3]
MHY1355	*MATα his3 leu2-3,112 ura3 trp1-1 nat1-5::LEU2 pup1Δ::leu2::HIS3* [pRS316-PUP1]
The following strains are congenic with MHY500:	
MHY785	*doa3-Δ1::HIS3* [YCp50-DOA3]
MHY1026	*pup1Δ::leu2::HIS3* [pRS316-PUP1]
MHY1376	*pup1Δ::leu2::HIS3* [YCplac22-pup1-T30A]
The following strains are congenic with MHY501:	
MHY784[c]	*doa3-Δ1::HIS3* [YCp50-DOA3]
MHY803[c]	*doa3-Δ1::HIS3* [YCplac22-DOA3-His_6]
MHY952[d]	*doa3-Δ1::HIS3* [YCplac22-Ub-doa3ΔLS-His_6] [YEplac181-DOA3LS]
MHY973[d]	*doa3-Δ1::HIS3* [YCplac22-Ub-doa3ΔLS-T76A-His_6] [YEplac181-DOA3LS]
MHY1031[e]	*pre3-Δ2::HIS3* [pRS316-PRE3]
MHY1051	*doa3-Δ1::HIS3 pre3-Δ2::HIS3* [YCp50-DOA3] [pRS316-PRE3]
MHY1157	*pre3-Δ2::HIS3* [YCplac22-pre3-T20A]
MHY1173	*pre3-Δ2::HIS3 pup1Δ::leu2::HIS3* [pRS316-PRE3] [pRS316-PUP1]
MHY1175	*doa3-Δ1::HIS3 pup1Δ::leu2::HIS3* [YCp50-DOA3] [pRS316-PUP1]
MHY1177	*pre3-Δ2::HIS3 pup1Δ::leu2::HIS3* [pRS317-PUP1] [YCplac22-PRE3]
MHY1178	*pre3-Δ2::HIS3 pup1Δ::leu2::HIS3* [pRS317-pup1-T30A] [YCplac22-pre3-T20A]
MHY1325	*pup1Δ::leu2::HIS3* [pRS316-PUP1]
MHY1326[f]	*ump1-Δ2::LEU2*
MHY1327[f]	*ump1-Δ3 doa3-Δ1::HIS3* [YCplac22-Ub-doa3ΔLS-His_6]
MHY1471	*nat1-4::LEU2 ump1-Δ3 doa3-Δ1::HIS3* [YCplac22-Ub-doa3ΔLS-His_6]
MHY1493	*pre3-Δ2::HIS3 pup1Δ::leu2::HIS3* [YCplac22-Ub-pup1ΔLS] [pRS315-Ub-pre3ΔLS]
MHY1506	*pup1Δ::leu2::HIS3* [YCplac22-PUP1-HA_2]
MHY1507	*pup1Δ::leu2::HIS3* [YCplac22-M-pup1ΔLS-HA_2]
MHY1513	*doa3-Δ1::HIS3 pup1Δ::leu2::HIS3* [pRS315-DOA3-His_6] [YCplac22-PUP1]
MHY1514	*doa3-Δ1::HIS3 pup1Δ::leu2::HIS3* [pRS315-DOA3-His_6] [YCplac22-Ub-pup1ΔLS]
MHY1515	*doa3-Δ1::HIS3 pre3-Δ2::HIS3* [YCplac22-DOA3-His_6] [pRS315-PRE3]
MHY1516	*doa3-Δ1::HIS3 pre3-Δ2::HIS3* [YCplac22-DOA3-His_6] [pRS315-Ub-pre3ΔLS]

[a]Chen *et al*. (1993).
[b]Mullen *et al*. (1989); MHY691 is identical to AMR1.
[c]Chen and Hochstrasser (1995).
[d]Chen and Hochstrasser (1996).
[e]Arendt and Hochstrasser (1997).
[f]Ramos *et al*. (1998); MHY1326 and MHY1327 are identical to JD60 and JD163, respectively.

proteasome biogenesis. The Doa3 subunit, in addition to having the most important protease active site, also has the only essential propeptide. Although *pup1ΔLS* and *pre3ΔLS* mutants have only minor growth defects, their study has allowed the elucidation of two general roles played by eukaryotic β-subunit propeptides in the production of active proteasomes. First, *pup1ΔLS* and *pre3ΔLS* mutants display defects in proteasome assembly, indicating a function for the propeptides of Pup1 and Pre3 in this process. An overlap in the function of these two propeptides is suggested by the enhanced growth defect of the *pup1ΔLS pre3ΔLS* double mutant. Secondly, each propeptide shields the amino group of the N-terminal Thr from cytosolic acetyltransferases until it is sequestered in the proteasome interior. This was shown most thoroughly for the Pre3/β1-subunit, but the data on Doa3/β5 and Pup1/β2 strongly suggest that this role is general. The specific inhibition of peptidase activities by N^{α}-acetylation also offers biochemical evidence for the suggestion from structural studies that the α-amino group is important for catalysis.

Acetylation of Thr1 in propeptide-deletion mutants

The N-terminal Thr residue of each of the catalytically active proteasome β-subunits appears to be susceptible to inactivation by N^{α}-acetylation if the subunit is synthesized without its propeptide. Nat1, together with Ard1, comprises the major N^{α}-acetyltransferase in yeast, and prefers sub-

strates with a small N-terminal residue, particularly Ser, Ala, Gly and Thr (Huang *et al*., 1987; Lee *et al*., 1990). By removing the propeptide of each subunit genetically, we have made the active β-subunits into good substrates for this enzyme.

Although doa3ΔLS, pup1ΔLS and pre3ΔLS all have N-terminal Thr residues, only pre3ΔLS appears to be nearly 100% acetylated. *In vitro* experiments using yeast or rat acetyltransferases and peptide substrates indicate that residues downstream of the terminal residue influence the degree of N^{α}-acetylation of the protein (Dixon and Woodford, 1984; Lee *et al*., 1990). Likewise, introduction of point mutations near the N-terminus of a protein can change its acetylation state *in vivo* (Tsunasawa *et al*., 1985). The residues following Thr1 in the active proteasome β-subunits are not identical between the three subunits (Figure 1A), and our data suggest that a threonine in the second position, as is found in Pup1 and Doa3, might disfavor N-terminal acetylation relative to a serine in that position.

Proteases are commonly synthesized with an N-terminal propeptide that inhibits protease activity, thereby preventing unregulated activation of the protease (Khan and James, 1998). We have shown that the propeptides of proteasome β-subunits play an opposite role, namely to protect β-subunits from inactivation by cellular enzymes. Perusal of the SWISS-PROT database shows that a number of proteins with an N-terminal threonine are N^{α}-acetylated in multicellular organisms, suggesting that β-subunit propeptides could protect proteasome subunits from N^{α}-acetylation in these species as well. More generally, there may be a variety of proteins that are synthesized with N-terminal pro-domains at least in part as a means to prevent N^{α}-acetylation. The *Thermoplasma* proteasome β-subunit has an eight-residue propeptide that is completely dispensable for proteasome assembly and activity when *Thermoplasma* particles are synthesized heterologously in *Escherichia coli*. However, the latter eubacterium has little N^{α}-acetyltransferase activity (Bradshaw *et al*., 1998), so study of the *Thermoplasma* propeptide-deletion mutant in its natural host may show that it too is subject to acetylation and catalytic inactivation. It is not yet known whether archaea such as *Thermoplasma* have N^{α}-acetyltransferases that could modify an N-terminal Thr, though sequence analysis has turned up possible homologues of Ard1 in two archaeal species (Bradshaw *et al*., 1998).

The α-amino group is essential for catalysis

The *Thermoplasma* proteasome crystal structure suggested that the active site contained a catalytic residue arrangement reminiscent of that of some serine proteases, with the hydroxyl group of Thr1 acting as the catalytic nucleophile, Lys33 polarizing the hydroxyl and Glu17 polarizing Lys33 (Löwe *et al*., 1995; Seemüller *et al*., 1995). All three of these residues are essential for both propeptide processing and peptidase activity (Seemüller *et al*., 1996). However, Lys33 appeared to be charged in the structure, and it was therefore regarded as unlikely to act as proton acceptor in catalysis (Löwe *et al*., 1995). Likewise, Lys33 is thought to be fully engaged in other hydrogen and ionic bonds during autolysis (Ditzel *et al.*, 1998). The only other basic group in the vicinity of the Thr1 hydroxyl was the α-amino group. This group is positionally and chemically suitable for the proton acceptor role, and could be seen to hydrogen bond to the hydroxyl side chain of Thr1 through a bridging water molecule in the higher resolution yeast structure (Groll *et al*., 1997).

Our data provide the first biochemical evidence for the importance of a free α-amino group in proteasome-mediated catalysis. By chemically modifying the α-amino group of pre3ΔLS through *in vivo* acetylation, we see that Pre3 peptidase activity is abolished, presumably because the resulting amido nitrogen is no longer suitable as a proton acceptor. To investigate the alternative possibility that the acetyl group perturbed the structure of the active site pocket, we modeled an acetyl group onto the mature end of the Pup1 subunit in the yeast 20S proteasome structure (PDB accession No. 1RYP), followed by local energy minimization (the Pre3 and Doa3 active sites are mutated in the proteasome used for this crystal structure). The acetyl group can be accommodated in the active site without displacing residues that bind substrate or interact with the hydroxyl of Thr1 (data not shown). Nevertheless, we cannot completely exclude the possibility that in other rotational conformations, an acetyl group could inactivate β-subunits by occluding substrate access or perturbing the structure of the active site.

β-subunit propeptides in proteasome assembly

Propeptides do more than allow catalytic site competence by protecting the α-amino group from acetylation. This is suggested by the poorer growth of the propeptide-deletion mutants relative to the corresponding catalytically inactive Thr to Ala mutants (Chen and Hochstrasser, 1996; Arendt and Hochstrasser, 1997). Were these propeptides necessary only to ensure full activity of their respective subunits, one would have expected the *doa3ΔLS* and *pup1ΔLS* strains to grow better than the *doa3-T76A* and *pup1-T30A* mutants since the propeptide-deletion strains retain more peptidase activity than do the Thr to Ala mutants. Additionally, we note that a *nat1* deletion does not rescue the lethality of the *doa3ΔLS* strain (data not shown). This result indicates that the Doa3 propeptide has an essential function apart from any effect it may have on the chymotrypsin-like active sites. Similarly, given the greater growth deficit of the *pre3ΔLS pup1ΔLS* mutant relative to that of the *pre3-T20A pup1-T30A* strain, the Pup1 and Pre3 propeptides must have important roles aside from their contributions to local catalytic site formation.

The pathway of proteasome assembly has received only modest attention, and the intermediates that are formed and the roles of accessory proteins and various proteasome subunits in this process are just beginning to be characterized. Mammalian proteasome intermediates with sedimentation constants of 13S, 15S and 16S have been described (Frentzel *et al*., 1994; Yang *et al*., 1995; Schmidtke *et al*., 1997). The 13S and 15S intermediates appear to correspond to half-proteasomes, with a full α-subunit ring and a ring of (unprocessed) β-subunits, although not all of the β-subunits may be present in the most abundant of these intermediate species (Nandi *et al*., 1997). A similar half-proteasome intermediate is also detectable in yeast (Ramos *et al*., 1998), and in the *in vitro* assembly of eubacterial *Rhodococcus* proteasomes (Zühl *et al*., 1997). The 16S particle appears to be a complete

but inactive proteasome in which β-subunit propeptides and other proteins are still present (Schmidtke *et al*., 1997).

The propeptides of Pup1 and Pre3 are not essential for proteasome assembly; however, the *pre3ΔLS* and *pup1ΔLS* mutants do display subtle defects in proteasome biogenesis. In pulse–chase measurements of Doa3 processing in the *pre3ΔLS* background, aberrant degradation products of Doa3 are produced *in vivo* within 30 min (Figure 3C). These Doa3 fragments were associated with complexes smaller than 20S proteasomes at steady state, and are likely to be off-pathway complexes accumulated due to the absence of the Pre3 propeptide. Were these complexes able to assemble into mature 20S proteasomes, we would have expected to detect the aberrant bands in proteasome-containing fractions as well. The small size of these complexes suggests that the Pre3 propeptide exerts an influence early in the assembly pathway.

The *pup1ΔLS* mutant, by contrast, accumulates what seems to be a normal processing intermediate of Doa3 (Figure 3C). Deletion of the proteasome assembly factor Ump1 leads to a severe slowdown in Doa3 processing and generation of a processing intermediate of similar mobility (Ramos *et al*., 1998). Perhaps a structural rearrangement, coordinated in part by Ump1, is required at the end of proteasome assembly to allow the auto-processing of propeptides. If the Pup1 propeptide were also involved at this late step, its deletion would be expected to slow the rate of Doa3 processing.

We conclude that all three β-subunit propeptides participate in yeast proteasome biogenesis. While the β-subunit propeptide of the much simpler *Thermoplasma* proteasome does not seem to contribute to proteasome assembly (Zwickl *et al*., 1994), propeptides may commonly assist in assembling more complicated proteasomes. The eubacterial *Rhodococcus* proteasome, for example, contains two α- and two β-subunits. Even at this modest level of subunit complexity, the organism makes use of β-subunit propeptides that are substantially larger than that of *Thermoplasma*, and their deletion severely retards *in vitro* assembly (Zühl *et al*., 1997). In the yeast proteasome, which has seven different α-subunits and seven different β-subunits, all three propeptides participate in assembly, although their contributions are distinct. These data suggest that the complexity of the subunit structure of a proteasome and the assembly role borne by its β-subunit propeptides may be directly related. As proteasome biogenesis became more complicated, assembly-active propeptides may have become necessary to help orchestrate and/or regulate the assembly pathway. It is likely that all eukaryotic β-subunit propeptides will eventually be found to have a role in proteasome assembly, though the steps at which they act and their exact contributions to the process may vary.

Materials and methods

Yeast and bacterial media and methods

Yeast rich (YPD) and minimal (SD) plates were prepared as described, and standard methods were used for genetic manipulation of yeast. *Escherichia coli* strain JM101 was used, and standard methods were employed for recombinant DNA work (Ausubel *et al*., 1989).

Construction of mutant and epitope-tagged proteasome alleles

pRS317-PUP1 was made by inserting the *Xba*I fragment from YCplac22-PUP1 (Arendt and Hochstrasser, 1997) into the *Xba*I site of pRS317 (Sikorski and Boeke, 1991). YCplac22-PRE3 was constructed by ligating the *Xba*I–*Xho*I fragment from p15E3 (Heinemeyer *et al*., 1997) into the *Xba*I–*Sal*I sites of YCplac22 (Gietz and Sugino, 1988). pRS317-pup1-T30A was made by inserting the *Eco*RI fragment from YCplac22-pup1-T30A (Arendt and Hochstrasser, 1997) into pRS317. pRS315-DOA3-His_6 was constructed by ligating the *Bam*HI–*Hin*dIII fragment from YCplac22-DOA3-His_6 (Chen and Hochstrasser, 1996) into pRS315 (Sikorski and Hieter, 1989).

For construction of Ub-pup1ΔLS and Ub-pre3ΔLS, a fragment containing the *CUP1* promoter and ubiquitin gene was first amplified from YEp96 (Ellison and Hochstrasser, 1991), with the addition of a *Kpn*I site to the 3′ end of the ubiquitin coding region, and then ligated into TA cloning vector pCRII (Invitrogen) to create pTA-CUP1-Ub. The *Kpn*I site in the pCRII polylinker was 5′ to the *pCUP1* sequence. The *PUP1* ORF contains two *Kpn*I sites: one at the propeptide processing site and one downstream of it. The downstream site was eliminated in YCplac22-PUP1 by a silent mutation using the QuikChange mutagenesis kit (Stratagene) to yield YCplac22-PUP1ΔKpnI. This construct was then cut with *Kpn*I, removing a fragment containing both the endogenous promoter and propeptide, and the *Kpn*I fragment from pTA-CUP1-Ub was ligated in, yielding YCplac22-Ub-pup1ΔLS. Similarly, *PRE3* contains *Kpn*I sites both at the propeptide processing site and downstream of it. The 5′ end of the *PRE3* coding region from pRS315-PRE3 (p15E3) was cut out with *Sac*I and *Pst*I, and ligated into YCplac22. This construct was then cut with *Kpn*I, and the *Kpn*I fragment of pTA-CUP1-Ub was inserted. The resulting Ub-pre3ΔLS-5′ fragment was excised with *Sac*I–*Pst*I, leaving the endogenous promoter behind, and ligated back into *Sac*I–*Pst*I-cut pRS315-PRE3, yielding pRS315-Ub-pre3ΔLS. YCplac22-Ub-pre3ΔLS was made by inserting the *Bam*HI–*Nsi*I fragment from pRS315-Ub-pre3ΔLS into the *Bam*HI–*Pst*I site of YCplac22. The *CUP1* promoter is copper inducible, but also allows a high level of constitutive expression; experiments described here were conducted without the addition of copper to the medium.

Double HA tags followed by the *CYC1* terminator were added to the 3′-termini of *PUP1* and *PRE3* using plasmids pJD508 and pJD498 (gifts of J.Dohmen). For making YCplac22-PUP1-HA_2, the 3′ portion of *PUP1-*HA_2 from pJD508 was exchanged with the 3′ end of *PUP1* from YCplac22-PUP1 by a *Xho*I–*Hin*dIII digest. To make YCplac22-Ub-pup1ΔLS-HA_2, *Ub-pup1ΔLS* was first subcloned into YEp195 (Gietz and Sugino, 1988) by a *Bam*HI–*Hin*dIII digest. The double HA tag was added to the 3′ end by exchanging *Bst*XI–*Hin*dIII fragments with pJD508, and the resulting *Ub-pup1ΔLS-*HA_2 construct was returned to YCplac22 with a *Sac*I–*Hin*dIII digest. For making HA_2-tagged Pre3, pJD498 was cut with *Pfl*MI to direct integration into the *PRE3* locus, and transformed into MHY501. Integrants were checked by anti-HA Western blotting. The tagged gene was then amplified from genomic DNA, and ligated into a TA-cloning vector (pGEM-T EZ, Promega). The tagged portion of the gene was then inserted in place of the 3′ ends of pRS315-PRE3 and pRS315-Ub-pre3ΔLS by *Nsi*I–*Pst*I digests. YCplac22 variants of pRS315-PRE3-HA_2 and pRS315-Ub-pre3ΔLS-HA_2 were made by subcloning *Hin*dIII–*Sac*I fragments into the corresponding sites in YCplac22.

YCplac22-M-pup1ΔLS-HA_2 and YCplac22-M-pre3ΔLS-HA_2 were made by two-step PCR, utilizing YCplac22-PUP1-HA_2 and YCplac22-PRE3-HA_2 as templates. Oligonucleotides were designed to delete the propeptide coding sequences. The final PCR products were ligated into the pGEM-T EZ TA cloning vector and then subcloned into YCplac22 by *Eco*RI digests. Sequencing of these constructs revealed that the *M-pup1ΔLS-*HA_2 open reading frame contained two conservative mutations: V26I and A52T. Because the mutant behaved almost identically to Ub-pup1ΔLS, we did not remake the allele.

Construction of yeast strains

Yeast strains used in this study are listed in Tables I and II. MHY1026 was a segregant from a cross between MHY998 (Arendt and Hochstrasser, 1997) and MHY500, and contained a *HIS3* marker only at the *pup1* locus. MHY1325 was derived from a cross between MHY1026 and MHY501. MHY1051 was constructed by crossing MHY784 and MHY1031, and choosing one of the two his^+ segregants of a non-parental ditype tetrad. MHY1175 and MHY1173 were constructed by crossing MHY785 and MHY1026, and MHY1026 and MHY1031, respectively, followed by sporulation.

Strains with chromosomal deletions of both *NAT1* and *PRE3* or *NAT1* and *PUP1* were constructed by crossing MHY691 to MHY1325 and to MHY1031, respectively. MHY1471 was constructed by transforming MHY1327 with the *nat1-4::LEU2 Bgl*II–*Bam*HI fragment from pJM118 (provided by R.Sternglanz).

All other strains were constructed by plasmid shuffling using the appropriate deletion strains and listed plasmids. After selection for transformants on the appropriate drop-out media, loss of wild-type genes carried on *URA3*-marked plasmids was selected for by streaking on plates containing FOA (Boeke *et al*., 1984).

Glycerol gradient analysis and peptidase activity assays of 20S proteasomes

Protein fractionation and peptide hydrolysis assays were performed essentially as described (Chen and Hochstrasser, 1995; Arendt and Hochstrasser, 1997). Protein amounts in each extract were determined by the Bradford method. Equal amounts of total protein were then loaded onto 10–40% glycerol gradients and centrifuged at 250 000 *g* for 17.5 h.

Pulse–chase and immunoblot analyses

Pulse–chase analysis was carried out as described (Chen *et al*., 1993). Immunoprecipitations were carried out with anti-α2 (Chen *et al*., 1993), anti-β-galactosidase (Cappel) or anti-LMP7-His_6 antibodies (gift of Y.Yang), which detects Doa3-His_6. For detection of HA-tagged proteins, the 16B12 monoclonal antibody (Babco, Richmond, CA) was used, followed by ECL detection (Amersham). 20S proteasome antigens were detected with antibodies raised against yeast 20S proteasomes (gift of K.Tanaka), followed by ECL detection or by binding to [^{125}I]protein A (NEN) and PhosphorImager analysis and quantitation (Molecular Dynamics).

Purification of 20S proteasomes and 2-D gel analysis

20S proteasomes from *Ub-pre3ΔLS* strain MHY1377 and *nat1Δ Ub-pre3ΔLS* strain MHY1373 were purified essentially as described previously (Chen and Hochstrasser, 1995) with the following exceptions: (i) a 1.6 cm×22 cm octyl-Sepharose 4FF column was used in the first step; and (ii) active fractions from the DEAE–Sepharose column were applied to a Superose 6 column after resuspending the 70% ammonium sulfate pellet in 500 μl 20S proteasome buffer (20 mM Tris–HCl pH 8.0, 1 mM EDTA, 150 mM NaCl, 10% glycerol). Proteasomes were eluted from this column with 20S proteasome buffer, and active fractions were pooled and precipitated with acetone. Pellets were resuspended at 1 mg/ml in IEF buffer and stored at –80°C.

Purified proteasome subunits were separated on isoelectric focusing tube gels and then by 12.5% SDS–PAGE (O'Farrell, 1975). Proteins were transferred to sequencing grade PVDF membranes (Millipore), and stained with Coomassie Blue. Pre3-containing spots were then cut out and sent for N-terminal sequence analysis.

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