The Active Sites of the Eukaryotic 20 S Proteasome and Their Involvement in Subunit Precursor Processing*

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The 26 S proteasome is the central protease involved in ubiquitin-mediated protein degradation and fulfills vital regulatory functions in eukaryotes. The proteolytic core of the complex is the 20 S proteasome, a cylindrical particle with two outer rings each made of 7 different α-type subunits and two inner rings made of 7 different β-type subunits. In the archaebacterial 20 S proteasome ancestor proteolytically active sites reside in the 14 uniform β-subunits. Their N-terminal threonine residues, released by precursor processing, perform the nucleophile attack for peptide bond hydrolysis. By directed mutational analysis of 20 S proteasomal β-type proteins of Saccharomyces cerevisiae, we identified three active site-carrying subunits responsible for different peptidolytic activities as follows: Pre3 for post-glutamyl hydrolyzing, Pup1 for trypsin-like, and Pre2 for chymotryptsin-like activity. Double mutants harboring only trypsin-like or chymotryptsin-like activity were viable. Mutation of two potentially active site threonine residues in the Pre4 subunit excluded its catalytic involvement in any of the three peptidase activities. The generation of different, incompletely processed forms of the Pre4 precursor in active site mutants suggested that maturation of non-active proteasomal β-type subunits is exerted by active subunits and occurs in the fully assembled particle. This trans-acting proteolytic activity might also account for processing intermediates of the active site mutated Pre2 subunit, which was unable to undergo autocatalytic maturation.

A 20 S proteasome ancestor was isolated from the archaebacterium Thermoplasma acidophilum which exhibits an electron microscopic structure like the eukaryotic proteasome core but a much simpler subunit complexity. Extensive structural studies on this complex (8–11) were completed by its x-ray crystallographic resolution (12). Two related subunits, α and β, form a stack of four heptameric rings, whereby the two outer rings are composed of α-subunits and the two inner rings of β-subunits. Four narrow gates arranged along the cylinder axis give rise to three cavities within the particle.

Analysis of the yeast 20 S proteasome (13–16) suggested that the eukaryotic particle contains 14 different but related subunits, encoded by 7 α-type and 7 β-type genes. These findings together with immunoelectron microscopic studies on mammalian 20 S proteasomes (17, 18) implied an architecture in which an ordered array of each 7 different α-type and 7 different β-type subunits is present in the two outer and in the two inner proteasomal rings, respectively. In vertebrates the subunit complexity is further extended by the fact that three of the constitutive β-type subunits can be replaced by closely related, γ-interferon inducible subunits that improve the function of proteasomes in the major histocompatibility complex class I-coupled antigen presentation pathway (reviewed in Refs. 5 and 19).

The eukaryotic 20 S proteasome has at least three different activities against synthetic peptide substrates as follows: a chymotryptsin-like, a trypsin-like, and a peptidyl-glutamyl peptide-hydrolyzing (PGPH) activity (20, 21). Yeast mutants defective in different peptidase activities of the 20 S proteasome had been isolated and were shown to carry alterations in distinct β-type subunits each (15, 22–24). The resulting assumption that β-type subunits contain the proteolytically active sites was substantiated by the x-ray structure determination of the Thermoplasma proteasome (12). This study as well as mutational analysis (25) identified the N-terminal threonine residue of the β-subunit, which becomes liberated by precursor processing during proteasome assembly (26), as the central amino acid necessary for proteolysis. This classifies the archaeabacterial β-subunit as a threonine protease within the family of N-terminal nucleophile hydrolases (27). The N-terminal threonine, Thr-β1, acts via its hydroxyl group as nucleophile in peptide bond hydrolysis and presumably is assisted by its own amino group as proton acceptor. In addition, Lys-β33 was found to be central for catalysis (12, 25), participating either indirectly by stabilizing and orienting active site residues or directly via its ε-amino group acting as proton acceptor for the Thr-β1 hydroxyl group. Both residues are necessary not only for external peptide cleavage (25) but also for the autocatalytic processing of the β-subunit (28). In the latter process, the

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1 The abbreviations used are: PGPH, peptidyl-glutamyl peptide-hydrolyzing; Cbz, carbobenzoxyl; βGal, β-galactosidase.
The tools for site-directed mutagenesis of the proteasomal genes by the megaprimer method are summarized. Mutated codons in the mutagenic primers are in italics and nucleotide exchanges are underlined.

| Mutant allele | Template plasmid (gene; insert fragment in pRS315) | Mutagenic primer (5’-3’) | Introduced changes of restriction sites | Resulting plasmid |
|---------------|-----------------------------------|-----------------|-------------------------------------|------------------|
| pre4-T34A     | p15-E4 (PRE4; 1.40-kbp Ecl136I/HindIII)         | GGTGCTGGCCCACTTAACACCAT | New Fpl site | p15-E4T34A |
| pre4-T42A     | p15-E4 (PRE4; 1.40-kbp NruI/SnaBl)              | GAGGCAAGACCTCTGATCTACCA | Loss of RsaI site | p15-E4T42A |
| pre3-T20A     | p15-E3 (PRE3; 1.42-kbp NruI/SnaBl)              | GCTCATGCTGATGATATAAGA | Loss of KpnI site | p15-E3T20A |
| pup1-T30A     | p15-P1 (PUP1; 1.77 kbp)                        | CCAGGTCAGCACCATTTGTA | Loss of KpnI site | p15-P1T30A |
| pre2-T76A     | p15-E2 (PRE2; 1.33 kbp, upstream end: BamHI)  | GTAGCCACATGTGCGATCTTGG | Loss of RsaI site | p15-E2T76A |
| pre2-T76S     | derived from exonuclease digestion,            | GTAGCCATGTGCGATCTTGG  | Loss of RsaI site | p15-E2T76S |
| pre2-K108A    | downstream end: BamHI                      | AACGGCTCTCACAGTTTGAAGAG | New HoeHI site | p15-E2K108A |
| pre2-K108R    |                                        | AACGGCTCTCACAGTTTGAAGAG | New CfoI site | p15-E2K108R |

See text for details.

a amino group of Lyn-β33 must be directly involved in catalysis since the amino function of Thr-β1 is still blocked in the precursor β-subunit.

N-terminal threonine residues are also found in the recently discovered eubacterial proteasomes (5) and in some of the eukaryotic proteasomal β-type subunits. A conserved catalytic mechanism in archaeobacterial and eukaryotic proteasomes was first implicated through the concomitant binding of the high, specific proteasome inhibitor lactacystin to the N-terminal threonine of the mammalian β-type subunit X/MB1 (29). Although N-terminal threonine modification by lactacystin was not detected in other mammalian β-type subunits, all three main peptidolytic activities were inhibitable by this compound.

A recent mutational analysis of the yeast subunit Pre2/Dea3, a homologue of X/MB1, showed that this subunit type indeed represents a threonine protease and is correlated with chymotrypsin-like peptidase activity (30). Pre2 also undergoes autocatalytic activation by removal of its propeptide, but in contrast to the archaeobacterial β-subunit, deletion of the Pre2 propeptide was lethal. Interestingly, viability was restored by providing the Pre2 propeptide in trans. Mutations of putative active site residues in the mammalian LMP2 subunit were found to prevent formation of the mature protein, but they did not interfere with another N-terminal cleavage in the LMP2 precursor at a position 8–10 residues upstream of the wild-type LMP2 processing site (31). This led to a model of an ordered two-step processing mechanism.

Using a more extensive genetic approach in yeast we prove here that three eukaryotic 20 S proteasomal β-type subunits represent N-terminal threonine proteases, and we show that the three proteasomal peptidase activities can clearly be ascribed to active centers in the yeast β-type subunits Pre3, Pup1, and Pre2. Moreover, analysis of the N-terminal processing of the non-active β-type subunit Pre4 in proteasomal active site mutants reveals an in vivo proteolytic action of the three threonine protease subunits and implies that in wild-type proteasomes maturation of Pre4 is exerted by the next accessible active site subunit, Pup1, the ring-to-ring neighbor of Pre4.

**EXPERIMENTAL PROCEDURES**

**General Methods**—For all yeast manipulations and preparation of yeast growth media, protocols described in Refs. 32 and 33 were followed. Recombinant DNA work was carried out according to standard procedures (32).

**Generation of Mutant Alleles**—Site-directed mutagenesis was performed using the PCR-based megaprimer method (Ref. 34) (Table I). Briefly, derivatives of the shuttle vector pRS315 (35) containing the respective proteasomal genes served as templates for a first PCR with a mutagenic primer and the appropriate one of two “outside” primers complementary to vector regions flanking the genomic inserts. The resulting product served as megaprimer for a second PCR together with the other outside primer yielding the full-length mutant gene. Suitable restriction fragments containing the mutated site were then exchanged against the corresponding wild-type fragments in the original pRS315 derivatives. The entire regions of the PCR-derived fragments were sequenced to verify the introduced mutation and to exclude unwanted additional mutations. Plasmid p15-P1 containing the PUP1 gene was created by inserting a 1.12-kbp EcoRI/XhoI fragment from pPHY97 (36) containing the major part of PUP1 into pRS316 (35). The missing native PUP1 promoter and the start of the coding region were then added by insertion of an Xhol-cut 0.65-kbp PCR fragment derived from genomic DNA with primers PUP1–5xho (5’-CCGCTCGAGCTCTCTCTTGGAAATCTC-3’) and PUP1–exoXho (5’-CTGAGACACTCTGTGGTCTCT-3’), yielding p16-P1. The 1.77-kbp insert was released by partial Xhol and BamHI digest and cloned into pRS315.

**Plasmid-dependent Putative Active Site Mutants (Plasmid Shufflers)**—The pRS315 derivatives harboring the different mutant alleles were introduced into the corresponding null mutant strains that were complemented by the respective wild-type genes on a URA3-marked plasmid (see Table II). 5’-Fluoroorotic acid selection was then used to identify descendants that had lost the URA3 marker. The disruption alleles of pre2, pre3, and pre4 have been described (see Table II). A p15::HIS3 null allele was constructed by cloning a p15-P1-derived 0.22-kbp XhoI/BclI fragment carrying 5’-flanking regions of PUP1 together with a 1.77-kbp BamHI fragment carrying the HIS3 gene into SalI/BamHI-cut pUC18 and subsequently inserting 3’-flanking regions of PUP1 on a 0.62-kbp AflII-filled-in-EcoRI fragment from p16-P1 between the SmaI and EcoRI sites yielding plasmid p18-P1::HIS3. A p15 knockout mutant was generated by one-step gene disruption (33) in strain WCG4a/::HIS3 and subsequent tetrad dissection.

**Chromosomal Integration of Mutant Alleles**—Exchange of proteasomal wild-type genes by respective mutant alleles in strain WCG4 was achieved by two-step gene replacement (33). Proteasomal mutant alleles were inserted into the integrative, URA3-marked plasmid pRS306 (35), and the resulting plasmids were linearized by cutting within the insert regions and introduced into WCG4. Correct chromosomal integration of the plasmids and maintenance of the mutations was verified among the transformants by genomic PCR and restriction analysis (see Table I). Descendants which by recombination had lost the plasmid sequences were then identified by 5’-fluoroorotic acid selection. Mutants and wild-type clones were distinguished by restriction analysis of genomic PCR products. Chromosomal introduction of the pre2-T76A allele was done analogously, using the diploid strain WCG4a::HIS3 yielding the heterozygous PRE2/pre2-T76A diploid WYH21.

**Overexpression of PRE2 Alleles**—Galactose-inducible PRE2 and PRE5—For overexpression experiments the PRE2 and PRE5 are cloned into the LEU2-marked high copied vector pRS425 (37) yielding p25-E2 and p25-E2T76A. Wild-type PRE2 was brought under control of the inducible GAL1 promoter by inserting a 0.92-kbp NspI/BamHI PRE2 fragment from p15-E2 into SpII/BamHI-cut p51-GAL/Sph, a modified version of plasmid pRS315-GAL (38) with an additional SpII site between the HindIII and SacI sites. By this the PRE2 translation-start ATG was positioned closely behind the GAL1 promoter sequences in the resulting plasmid p51-GAL-E2. Plasmid p15-GAL-E5 was constructed analogously by inserting a 0.83-kbp NspISacI PRE5 fragment into SpII/SacI-cut p15-GAL/Sph. Strain WYH220 (pre2a::HIS3 [p15-GAL-E2]) is a haploid descendant of a diploid pre2a::HIS3/PRE2 strain transformed with p15-GAL-E2. Accordingly,

2 P. Hieter, unpublished results.
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Table II Yeast strains used in this study

| Strain       | Relevant genotype                |
|--------------|----------------------------------|
| WCG4a        | MATa leu2-3,112 ura3 his3-11,15 Can6 GAL2 |
| WCG4a        | MATa leu2-3,112 ura3 his3-11,15 Can6 GAL2 |
| WCG4a/a      | WCG4a X WCG4a; diploid           |
| YWH10        | pup1A::HIS3 [p16-P1]             |
| YWH11        | pup1A::HIS3 [p15-P1T30A]         |
| YUS4         | pup1-T30A                        |
| YWH30*       | pre3A::HIS3 [p16-E3]             |
| YWH31        | pre3A::HIS3 [p15-E2T20A]         |
| YUS1         | pre3-T90A                        |
| YUS5         | pup1-T30A pre3-T20A              |
| YIH39–1/2b   | pre422::HIS3 [p16-E4]            |
| YWH41        | pre422::HIS3 [p15-E4T34A]        |
| YWH42        | pre422::HIS3 [p15-E4T42A]        |
| YUS2         | pre4-T42A                        |
| YUS3         | pre4-T42A                        |
| YWH215       | pre2A::HIS3 [p16-E2]             |
| YWH215       | pre2A::HIS3 [p15-E2T76S]         |
| YWH123       | pre2A::HIS3 [p15-E2K108A]        |
| YWH214       | pre2A::HIS3 [p15-E2K108R]        |
| YWH223       | pre2-K108A                       |
| YWH224       | pre2-K108R                       |
| YWH225       | pre2-T76S                        |
| YWH226       | pre2-K108R pre3-T20A             |
| YWH227       | pre2::P3-T76A                    |
| YWH228       | pre2::P3-T76A                    |
| YWH229       | pre3A::HIS3 [p15-GAL-E2]         |
| YWH222       | pre2::P3-T76A                    |
| YWH250*      | pre5A::HIS3 [p15-GAL-E5]         |
| YWH250       | pre5A::HIS3 [p16-GAL-E5]         |
| YWH250       | pre5A::HIS3 [p15-GAL-E5]         |
| YWH150       | pre5A::HIS3 [p15-GAL-E5]         |
| YWH200       | pre2::P8429                      |
| YWH201       | pre2::P8429                      |
| YWH202       | pre2::P8429                      |

* R. Gücel and W. Hilt, unpublished, disruption allele as in Ref. 24.
* M. Bernert and W. Hilt, unpublished, disruption allele as in Ref. 23.
* Unpublished, disruption allele as in Ref. 22.
* Unpublished, disruption allele as in Ref. 16.

strain YIH390 (pre5A::HIS3 [p15-GAL-E5]) was derived by sporulation of a pre5A::HIS3::PRE5 diploid harboring p15-GAL-E5. YWH22 (pre2::P3-T76A) transformed with p15-GAL-E2 led to YWH221 and YWH222 after sporulation and tetrad dissection on YPGal plates. For shut-off experiments strains transformed with p15-GAL-derived plasmids were pre-grown in 2% galactose containing synthetic complete medium without leucine, washed with water, and divided into fresh medium for colony overlay assays applied to indicate in situ proteasomal peptidase activity against the substrates N-Clsg-Gly-Gly-Leu-p-nitroanilide and N-Suc-Leu-Leu-Glu-β-naphthylamide have been described (15, 23). Trypsin-like proteasomal activity in yeast cells has been described (15, 23). Trypsin-like proteasomal activity in yeast cells was determined photometrically as described (15, 38) and related to the optical density of the cells employed in the test. Peptidase activity measurements with crude extracts, prepared in small scale according to Ref. 15, were done anolously. Large scale preparation of crude extracts and fractionation by gel filtration chromatography were carried out essentially as in Ref. 38. Measurement of β-galactosidase activity using o-nitrophenyl-β-d-galactoside in strains harboring plasmids expressing ubiquitin-X-β-galactosidase fusion proteins was done according to Ref. 39.

**Immunoblotting, Antibodies, Preparation of heat-denatured crude cell extracts (Fig. 6) or non-denatured cell extracts (Fig. 3), separation by SDS-polyacrylamide gel electrophoresis on 18% gels, and electrophoretic transfer to nitrocellulose paper (22) were done according to Ref. 39.**

**RESULTS**

**Candidate Active Site Forming β-Type Subunits of the Yeast 20 S Proteasome: Experimental Approach—**Assuming a conserved catalytic mechanism of proteasomes throughout the kingdoms of life a sequence comparison of eukaryotic β-type subunit sequences with the archaeobacterial β-subunit predicted that only a subset of the seven different β-type members may form proteolytically active sites (25). Among the seven β-type subunits from the Saccharomyces cerevisiae 20 S proteasome only four (Pre2, Pre3, Pup1, and Pre4) carry a threonine in a position homologous to Thr-β1 from Thermoplasma (Fig. 1). For Pre2 and Pre3, it was shown that such threonines form the N termini of the mature subunits after proteptide removal (40). Although not determined experimentally, the N terminus of the mature Pup1 subunit is expected to be formed also by the threonine aligning with Thr-β1, since for Pup1 homologues from higher eukaryotes this threonine was identified as N terminus by protein sequencing (41–43). In contrast, N-terminal sequence determinations for Pre4 homologues (41–43) predict cleavage of the Pre4 precursor at a threonine residue 8 amino acids upstream of the threonine which is equivalent to Thr-β1. In analogy to its mammalian N3 homologues, Pre4 is the only yeast β-type subunit lacking a lysine residue corresponding to Lys-β33 from Thermoplasma.

We mutated putative active site forming residues of the candidate subunits Pre2, Pre3, and Pup1 and focused on the effect of these alterations on the three well-known peptidase specificities of the 20 S proteasome. To clarify its possible involvement in these activities, we also investigated subunit Pre4, which does not fulfill all requirements for a threonine protease of the known type. Mutant alleles of the different subunit genes were first tested by plasmid shuffling for their ability to complement lethality of the respective null mutant. Non-lethal mutations were then introduced into their native chromosomal loci by replacement of the respective wild-type genes. This avoided unwanted effects associated with expression of plasmid-borne mutant alleles like up-regulation of plasmid copy number or abnormal gene expression levels due to lack of regulatory promoter sequences.

**Mutations of N-terminal Threonines in Pre4 Do Not Alter Proteasomal Peptidase Activities and Pre4 Processing—**The mature Pre4 subunit is derived from precursor processing, but the precise processing site is unknown. The molecular mass of mature Pre4 of about 24.5 kDa, as estimated from immunoblot analysis of purified disassembled yeast 20 S proteasome (Fig. 3), favors processing at Thr-34, the site equivalent to that determined for Pre4 homologues from other species (41–43, Fig. 1).

To test whether this presumed N-terminal threonine of Pre4

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3. M. Bernert and W. Hilt, unpublished results.
functions as nucleophile in peptide hydrolysis, we constructed the pre4-T34A allele encoding a Pre4 protein with Thr-34 mutated to alanine. Correspondingly, we also mutated Thr-42 located at the Thr-β position, since we could not completely rule out that Thr-42 forms the N terminus or that Thr-42 functions in catalysis without being N-terminally exposed. Expressed from a plasmid each mutant allele was able to confer viability to a pre4Δ deletion mutant. After introducing both mutations independently into the chromosomal PRE4 gene, the resulting mutant strains YUS2 (pre4-T34A) and YUS3 (pre4-T42A) were tested in an in situ overlay assay for their proteasomal peptidase activities (Fig. 2A). They were indistinguishable from those of an isogenic wild-type strain. A more thorough measurement using defined amounts of permeabilized cells yielded activities against all three peptide substrates which in the mutants differed from wild-type levels by not more than 20% (Table III). This was confirmed biochemically by the peptidase activity profiles obtained after fractionating crude extracts from both pre4Δ mutants by gel filtration (not shown).

In agreement with an almost unaltered proteolytic capacity of the pre4 mutant proteasomes was the growth behavior of both mutants. They grew like wild-type cells on rich medium (Table III) and on poor mineral medium (not shown) at the different temperatures tested.

From these data we conclude that the threonine residues Thr-34 and Thr-42 in Pre4 participate neither in the three proteasomal peptidase activities (Fig. 2) nor in the catalytically important residues Thr-1 and Lys-33 in Ta-beta. Conservation of these residues in the yeast proteins is indicated in bold letters. Double underlining marks residues mutated in this study. Mammalian homologues of the yeast proteins are named in the right column.

**Pre3 Bears the Catalytic Site for the PGPH Activity—Screening of Mutagenized Yeast Cells for Defective Proteasomal PGPH Activity**

Gene disruption was achieved by inserting into the chromosomal PRE4 gene, the mutant gene integrated into the natural chromosomal locus (YUS1) were indeed almost devoid of the proteasomal PGPH activity. A pre3-T20A mutant allele was constructed which on a centromeric plasmid complemented lethality of a pre3 deletion mutant. Strains having the pre3-T20A mutant gene integrated into the natural chromosomal locus (YUS1) were indeed almost devoid of the PGPH activity, when assayed in situ using the peptide substrate N-Cbz-Leu-Leu-Glu-β-naphthylamide (Fig. 2B and Table III). The chymotrypsin-like activity was nearly unaffected, and the trypsin-like activity was even enhanced in pre3-T20A cells. These results were reflected in the peptidase activity profiles obtained after gel filtration of wild-type and pre3-T20A mutant cell extracts (not shown). Here the PGPH activity peak in the 20 S proteasome region of around 700 kDa was completely absent in the mutant, but some PGPH activity was detectable in fractions of lower molecular mass of around 300 kDa. This activity was also seen in the wild-type profile as a shoulder connected to the 20 S proteasomal peak. From inhibitor studies (not shown) we conclude that this activity cannot be attributed to the proteasome or proteasomal subcomplexes.

Loss of the PGPH activity in the pre3-T20A mutant had no detectable effect on cell growth. Compared with a wild-type strain no difference in colony size was visible after growth of the mutant on rich (Table III) or poor medium (not shown) at different temperatures. This implied that vital cellular functions dependent on the action of the proteasome are maintained in the absence of the proteasomal PGPH activity.

A largely unimpaired proteolytic capacity of pre3-T20A mutant proteasomes was verified by measuring the steady state activity levels of short lived β-galactosidase derivatives (so-called N-end-rule substrates), which are known to be degraded by the proteasome (39, 44). Like the long lived derivative Ala-βGal, which is not a proteasomal substrate, the short-lived βGal variants Arg-βGal and ubiquitin-Pro-βGal exhibited rather similar activity levels in wild-type and isogenic pre3-T20A mutant cells (not shown), indicating that their turnover by the proteasome was unaffected by the loss of the PGPH activity. We conclude that the active site for proteasomal PGPH ac-
tivity resides in Pre3 and that this activity is dispensable for in vivo functions of the proteasome. This points to a redundancy in the action of proteasomal active sites.

_Pup1 Bears the Catalytic Site for the Trypsin-like Activity—_The gene encoding the β-type subunit Pup1 was found accidentally (36). So far no pup1 mutants have been described. The protein is predicted to be N-terminally processed by removal of a 29-residue prosequence yielding an N-terminal threonine which makes it a candidate active site carrying subunit. We constructed a centromeric plasmid carrying a _pup1-T30A_ allele that was able to complement the lethal phenotype caused by the chromosomal deletion of _PUP1_. Replacement of the chromosomal _PUP1_ wild-type gene by the _pup1-T30A_ allele led to mutant strains (YUS4) which exhibited a strong reduction of the _in situ_ trypsin-like activity, whereas the two other peptidase activities remained nearly at wild-type levels (Fig. 2B and Table III). In the activity profiles obtained after gel chromatographic fractionation of crude extracts from _pup1-T30A_ mutant cells, the proteasomal trypsin-like activity was reduced by 85% (Table III). No significant alterations in the PGPH and the chymotrypsin-like activity profiles were observed (not shown).

The _pup1-T30A_ mutation led to a rather surprising cell growth defect. At 30 and 37 °C, strain YUS4 grew like wild type, but at a low temperature of 15 °C growth of the mutant was drastically slowed (Table III). Evidence for a general proteasomal proteolysis defect caused by the _pup1-T30A_ mutation came from the determination of the steady state activities of short lived β-galactosidase substrates. Ubiquitin-Pro-βGal had a 2.5-fold higher activity and Arg-βGal even a 10-fold higher activity in YUS4 than in wild type, indicating that turnover of these substrates by the proteasome harboring the mutated Pup1 subunit is decreased.

These results clearly assign the active site responsible for the proteasomal trypsin-like activity to the β-type subunit Pup1, which so far had not been correlated with proteolytic activities of the proteasome. As for Pre3, neutralization of the active site in Pup1 is compatible with cell viability, but in contrast to the Thr-20 → Ala exchange in Pre3, the Thr-30 → Ala mutation in Pup1 negatively affects cell proliferation and turnover of short lived proteins.

_Yeast Cells Survive without PGPH and Trypsin-like Activities—_We created a double mutant (YUS5) that carries a combination of the _pre3-T20A_ and the _pup1-T30A_ alleles and thus lacks both the PGPH and the trypsin-like peptide activity (Fig. 2B and Table III). Remarkably, chymotrypsin-like proteasomal activity alone seems to be sufficient for cell survival. The phenotypes of the _pre3 pup1_ double mutant are somewhat stronger as compared with those observed for the _pup1-T30A_ single mutant. Growth at 15 °C was further slowed (Table III), and the activity of the short lived N-end-rule substrate Arg-βGal was enhanced to 14-fold of the wild-type level. Thus, the Thr-20 → Ala mutation in Pre3, which alone did not cause a phenotype, does affect cell viability when combined with another proteolytic defect in the proteasome.

_Pup1 Is Responsible for Pre4 Precursor Processing—_A recently introduced model for the subunit arrangement in the human 20 S proteasome (45) suggests the following topography of β-type subunits in the yeast complex (Fig. 4). The Pre3 and

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**Fig. 2. In situ assays for different proteasomal peptidase activities.** Patches of yeast cells chromosomally harboring the proteasomal mutant alleles as indicated were grown in triplicate on filters, permeabilized, and covered with gels containing different peptide substrates to assay trypsin-like (N-Cbz-Ala-Arg-4-methoxy-β-naphthylamide), chymotrypsin-like (N-Cbz-Gly-Leu-p-nitroanilide), or PGPH (N-succinyl-Leu-Leu-Glu-β-naphthylamide) proteasomal activities. After incubation for 3–5 h released fluorophores or chromophores were converted to visible dyes as indicated under “Experimental Procedures.” Wild-type cells were compared with strains mutated in potential active site threonines of Pre4 (A), strains mutated in the active site threonines of mature Pre3 or and Pup1 (B), and mutants expressing Pre2 variants with non-lethal active site mutations (C).
In situ peptidase activities and growth of putative proteasomal active site mutant strains

The indicated mutant alleles were chromosomally integrated. Substrates were the same as in legend to Fig. 2. Peptidase activity values are means of at least two measurements using two strains of opposite mating type. (Because of high background peptidase activities in the in situ assay the values for trypsin-like activity in pup1-T30A strains were calculated by integrating specific activities measured in gel chromatography fractions of the molecular mass range of between 100 and 1000 kDa). Sizes of single wild-type and mutant colonies grown for 2–4 days on rich medium at different temperatures are expressed in arbitrary units (+), –, no visible colonies.

| Strain | Relevant genotype | Peptidase activity (% of wild type) | Growth |
|--------|------------------|----------------------------------|--------|
|        |                  | PGPH Trypsin-like Chymotrypsin-like | 30 °C 15 °C 37 °C |
| WCG4   | Wild-type        | 100 100 100                       | ++ ++ ++ |
| YUS8   | pre3-T74A        | 80 113 110                        | ++ ++ ++ |
| YUS9   | pre4-T24A        | 93 97 100                         | ++ ++ ++ |
| YUS1   | pre3-T20A        | 7 164 89                          | ++ ++ ++ |
| YUS4   | pup1-T30A        | 85 15 86                          | ++ ++ ++ |
| YUS5   | pre4-T30A pre3-T20A | 3 20 95 | ++ + + |
| YWH23  | pre2-K108A       | 217 191 4                         | + + ++ |
| YWH24  | pre2-K108R       | 149 178 3                         | + + - |
| YWH26  | pre2-K108R pre3-T20A | 36 225 2  | ++ + - |
| YWH25  | pre2-T76S        | 149 189 113                       | ++ ++ - |

![Fig. 3. Anti-Pre4 immunoblot analysis of potential active site mutants in Pre4, Pre3, or/and Pup1.](Image)

Pup1 components are lying adjacent to each other in each of the two symmetry related β-type rings, such that the two Pre3 subunits are located opposite each other. Pre4 is the other neighbor of Pre3 within each ring, having contact with the Pup1 subunit across the rings. Thus, Pre4 is predicted to be in close proximity to both active site-carrying subunits Pre3 and Pup1. We investigated whether integrity of the active sites in Pup1 and/or Pre3 is necessary for normal N-terminal processing of Pre4 by immunoblot analysis of crude extracts from pre3 and pup1 single and double active site mutants. In the pre3-T20A sample, Pre4 is matured to the same size as in wild type (Fig. 3, lanes 3, 6, and 7). The corresponding Pre4 protein from pup1-T30A cells shows a slight increase in molecular mass corresponding to additional 3–4 amino acids (Fig. 3, lane 4). In the pre3-T20A pup1-T30A double mutant, a processed Pre4 fragment of even higher molecular mass is seen (Fig. 3, lane 5).

Obviously, Pup1 is involved in maturation of Pre4. The fact that the pre3-T20A mutation has a visible effect on Pre4 maturation only when combined with the pup1-T30A allele can be explained by the following model (Fig. 4). Pup1, located opposite site to Pre4, is responsible for processing of Pre4 to its wild-type form. When Pup1 is inactive, the Pre4 propeptide is cut somewhat upstream of the Pup1 processing site by the next accessible active site, located in Pre3. Since such cleavage by Pre3 is apparently not a prerequisite for cleavage by Pup1, inactivation of Pre3 alone has no effect on Pre4 maturation. When both Pup1 and Pre3 are inactive, there still occurs processing of the Pre4 precursor, however at a site located upstream of that used by Pre3. This cut must be exerted by the third type of active site subunit, Pre2, which lies a greater distance from Pre4 than Pup1 and Pre3 do. Cleavage by Pre2 is also not a prerequisite for the processing by Pup1, as active site mutations in Pre2 do not affect Pre4 maturation (not shown).

This model has several important implications. (i) N-terminal processing of non-active β-type subunits like Pre4 is exerted in trans by the active threonine protease subunits of the proteasome. (ii) This trans-processing event occurs between the two β-type subunit rings, i.e. in a fully assembled 20 S proteasome. (iii) The propeptides of non-active subunits can freely move around in the inner proteasomal cavity and can be cleaved unspecifically by all catalytic centers to which they gain access. The maximal length reduction of these propeptides is determined by the position of the closest active site.

Pre2 Bears the Catalytic Site for the Chymotrypsin-like Activity—The third candidate for a proteolytically active β-type subunit, Pre2, was expected to be responsible for chymotrypsin-like activity. At least six randomly generated mutant alleles of the PRE2 gene (also identified as PRG1 and DOA3; Refs. 40 and 46) had already been found to cause a defect in the proteasomal chymotrypsin-like activity (22, 40, 47). Recently, a directed mutational study clearly identified Pre2 as a proteasomal threonine protease subunit with chymotrypsin-like specificity (30). Our analysis of mutations in putative Pre2 active site residues is basically consistent with these data. However, details of our findings are different.

A pre2-T76A mutant allele coding for a Pre2 variant with the N-terminal threonine of the mature subunit exchanged to alanine was lethal in our wild-type strain background. (i) After introduction of a pre2-T76A carrying plasmid into a pre2Δ null mutant strain harboring the PRE2 wild-type gene on a URA3-marked plasmid, counterselection against URA3 yielded no viable clones. (ii) Sporulation of a diploid strain, heterozygously carrying the pre2-T76A mutant allele, gave rise to only two viable, PRE2 wild-type spore clones. The two non-viable pre2-T76A clones formed microcolonies consisting of up to 16 cells, a phenotype comparable to pre2Δ spores. Expression of the pre2-T76A mutant allele on a high copy plasmid in a wild-type strain had a dominant negative effect on cellular growth (not shown).

Both findings, lethality of the pre2-T76A mutation in the absence and growth inhibition by its overexpression in the presence of wild-type Pre2, could be explained either by loss of

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5 W. Heinemeyer, unpublished results.
an essential proteolytic function residing in Pre2 or by a disturbance of proteasome assembly and/or stability, due to defective processing of the Pre2 precursor. To distinguish between these possibilities, we performed a shut-off experiment with a strain carrying the pre2-T76A allele on the chromosome and harboring a plasmid with the PRE2 wild-type gene under control of the inducible GAL1 promoter. Cells pre-grown in galactose-containing medium were transferred into fresh galactose or repressing glucose medium. After 15 h, peptidase activities (Fig. 5) and the viability (not shown) of the galactose and glucose-grown cells were determined. In a pre2Δ control strain as well as in a pre5Δ control strain (deleted for an α-type proteosomal gene) all three peptidase activities were drastically decreased after glucose repression of the respective wild-type genes. In contrast, the glucose-grown pre2-T76A cells showed still nearly wild-type PGPH and trypsin-like activities but a clear drop in chymotrypsin-like activity (Fig. 5). Unlike the pre2Δ and pre5Δ strains, almost all of the pre2-T76A cells were viable after re-transfer to galactose medium (not shown). This implies incorporation of the mutated Pre2 subunit into assembling proteasomes, which causes loss of chymotrypsin-like activity. In contrast, depletion of the wild-type Pre2 or Pre5 subunit in the corresponding deletion mutants leads to loss of all activities and cell death, indicating stop of de novo proteasome formation.

In the β-subunit of Thermoplasma Lys-β33 is necessary for autocatalytic peptidase processing and for peptide cleaving activity of the mature subunit (25, 28). To show if the corresponding lysine in Pre2, Lys-108, has an equivalent function, we exchanged it to alanine or arginine. Both the pre2-K108A and the pre2-K108R mutant allele, when expressed from a centromeric plasmid, restored viability of a pre2Δ strain, although the cell growth was severely disturbed, especially at elevated temperatures. Chromosomal introduction of the mutations led to the same growth defects (Table III). In the in situ peptidase assay the mutants were almost completely devoid of proteasomal chymotrypsin-like activity, but they both exhibited strongly enhanced PGPH and trypsin-like activities (Fig. 2C and Table III). Loss of chymotrypsin-like activity in these pre2-K108 mutants clearly ascribes the active site responsible to the Pre2 subunit and predicts a similar role for Lys-108 in catalytic center formation as for Lys-β33 in the archaeobacterial proteasome. The increased trypsin-like and PGPH activities in the pre2-K108 mutants are most probably due to an elevated concentration of 20 S proteasomes.

Combination of the Lys-108 → Arg mutation in Pre2 with an inactivated Pre3 subunit was still compatible with viability. Growth of a pre2-K108R pre3-T20A double mutant was slower than that of the pre2-K108R single mutant, and trypsin-like peptidase activity was further increased (Fig. 2C and Table III). Thus, similar to the situation in a pre3-T20A pup1-T30A double mutant having only chymotrypsin-like activity, trypsin-like proteasomal activity is sufficient for survival. However, a cross between the pre2-K108R and the pup1-T30A mutants yielded no viable double mutant clones, indicating that PGPH activity alone is not sufficient for survival. This may be explained by a hierarchy among the three types of active sites, but we cannot completely exclude that proteasome assembly defects due to impaired maturation of the Pre2 and Pup1 precursors are responsible for lethality of a pre2-K108R pup1-T30A double mutant.

The β-subunit of the Thermoplasma proteasome showed an impaired peptidase activity when Thr-β1 was exchanged to serine (25). However, this mutation reduced the efficiency of autocatalytic propeptide processing (28), indicating a different geometry of the mature active site and the site acting in the self-maturation step. We constructed the corresponding pre2-T76S allele to check whether these findings would also apply to the yeast Pre2 subunit. Pre2-T76S mutants were viable and indeed showed chymotrypsin-like activity comparable to wild-type levels (Fig. 2C and Table III). On the other hand, they behaved similar to the pre2-K108 mutants in that they exhibited temperature sensitivity, slow growth at normal temperature, and a significant increase of the proteasomal PGPH and trypsin-like activities (Fig. 2C and Table III). Thus, although serine can substitute for threonine to retain chymotrypsin-like activity, this exchange leads to diminished cell viability.

As revealed by immunoblot analysis, maturation of the Pre2-Thr-76 → Ser subunit was not prevented, although an increased amount of unprocessed Pre2 precursor was detectable in pre2-T76S cell extracts as compared with wild-type cell extracts (Fig. 6, lanes 1, 4, and 5). In contrast, mutations in the conserved lysine residue of Pre2 lead to more severe defects in maturation. The Lys-108 → Ala mutation completely abolishes maturation of Pre2 to wild-type molecular size (Fig. 6, lane 3). However, a processing intermediate is formed. In analogy to Pre4 we propose that this intermediate is generated through cleavage by other active site containing subunits. As an intermediate of similar size is generated in pre2-K108R pre3-T20A double mutants (not shown), we suggest Pup1 to be responsible. The Lys-108 → Arg mutation also leads to generation of this intermediate but does not completely prevent normal maturation of Pre2 (Fig. 6, lane 2). This, however, does not lead to chymotrypsin-like activity. Apparently, arginine can partially
replace the conserved lysine for the processing event, but not for external peptide cleavage by the matured subunit.

**DISCUSSION**

**Proteolytic Centers in Eukaryotic 20 S Proteasomes**—Our analysis assigns the three well established peptidase activities of eukaryotic 20 S proteasomes to three distinct yeast proteasomal \( \beta \)-type subunits. Mutants expressing variants of Pre3, Pup1, and Pre2 with the N-terminal threonine residues replaced by alanine are defective in the PGPH, trypsin-like, and chymotrypsin-like activities, respectively. Furthermore, Lys-108 in Pre2, the counterpart of which contributes to active site formation in the Thermoplasma \( \beta \)-subunit, is also essential for chymotrypsin-like peptidase activity of the yeast protein. Thus, the three yeast subunits Pre3, Pup1, and Pre2 represent threonine proteases of the N-terminal nucleophile-hydrolase superfamily (27) containing active sites similar to those of the Thermoplasma proteasome. In mammals the homologues of yeast Pre3, Pup1, and Pre2 are found as pairs of interchangeable subunits. Replacement of the constitutive components \( \delta \gamma, \alpha \zeta, \) and \( \varepsilon \alpha \nu \) by the \( \gamma \)-interferon inducible subunits LMP2, MECL1, and LMP7 yields so-called immunoproteasomes (for review see Ref. 19) that generate peptides from intracellular antigens preferred for presentation by major histocompatibility complex class I molecules. Their improved function in antigen processing had been correlated with changes in cleavage activities against fluorogenic model peptides (48–53), although the processing had been correlated with changes in cleavage activity to Pre2. No inhibitor was bound to Pre4, which indeed contained the expected N-terminal extension of 8 residues starting with Thr-34. In addition, Groll and co-workers (58) postulated a new kind of active site at the C-terminal ends of short \( \alpha \)-helices that are present in all 7 \( \beta \)-type subunits and that together form an anulus surrounding the inner surface of each \( \beta \)-ring. This hypothesis is based on the finding that the N termini of both partially processed, non-active subunit neighbors Pre4 and Pre3 were situated at one of these \( \alpha \)-helices, indicating to the authors that cleavage had occurred at this site. However, this model of a precursor processing mechanism is in conflict with our genetic data that imply that the Pre4 precursor is cleaved by an active Pup1 subunit. We therefore consider this \( \beta \)-anulus not to be involved in subunit processing, and we also doubt a proteolytic function of this \( \beta \)-anulus in substrate degradation. From the character of the substrate binding pockets in the three threonine protease subunits, Groll et al. (58) postulated Pre2 to be responsible for both chymotrypsin-like and trypsin-like peptidase activity and the Pup1 pocket to be destined for binding of large neutral residues at the P1 position of substrates. This was also supported by the exclusive covalent binding of the specific proteasome inhibitor lactacyclin to the catalytic center of Pre2. In mammalian proteasomes lactacyclin inhibited both the chymotrypsin-like and the trypsin-like activities by modifying one subunit, the Pre2 homologue X/MBA1 (29). This contradicts our data that clearly assign these two different peptidase activities to one of two different subunits each, chymotrypsin-like activity to Pre2 and trypsin-like activity to Pup1. However, a residual trypsin-like activity of 15–20% is found in pup1-T30A single and in pup1-T30A pre3-T20A double mutant strains (Table III). This might be due to cleavage by the catalytic center in Pre2. Fenteany and co-workers (29) used a different substrate to measure trypsin-like activity to Pre2.
activity. Thus, overlapping substrate specificities of catalytic sites could in part be responsible for the dual inhibition.

The crystal structure of the yeast 20 S proteasome (58) explains our earlier mutant data that correlated two β-type subunits, Pre2 and Pre1, with chymotrypsin-like and two β-type subunits, Pre3 and Pre4, with PGPH activity. The mutations in the non-active Pre1 and Pre4 subunits affect contact regions to the neighboring active subunits Pre2 and Pre3, respectively, and are likely to disturb the conformation in their catalytic centers. Thus, the function of non-active β-type subunits may not reside in the formation of active centers, as previously discussed (15, 22, 45), but in binding and orienting substrate peptide chains in hydrophobic clefts allowing neighboring active site subunits to cleave peptide bonds. This cooperativity might support the processivity and the narrow size range of cleavage products characteristic for proteasomal proteolysis.

Our results point to the redundancy in proteolytic function of active proteasomal subunits. Loss of PGPH activity had no effect on cell viability, lack of trypsin-like activity had only moderate consequences, and also loss of the chymotrypsin-like activity in the pre2-K108A and pre2-K108R mutants allowed survival (Table III). Even one functioning active site can be sufficient to maintain vital proteasomal functions, as indicated by the viability of pre3 pup1 and pre3 pre2 double mutants. Why then have three different proteolytically active subunits evolved in eukaryotic cells? One obvious explanation is a complementation of the three active subunits with regard to their cleavage specificity, by this improving and ensuring the processivity during substrate degradation. A differentiation of cleavage site preferences in natural protein substrates, however, cannot simply be deduced from the classification of the three proteasomal peptidase specificities. For example, the Thermoplasma proteasome harboring only one type of active site with chymotrypsin-like peptidase activity can cleave also after basic and acidic amino acid residues in model protein substrates (59). Several other investigations (for example see Ref. 52) indicated that cleavage of peptide bonds depends not only on the residue in the P1 position but also on amino acids preceding the cleavage site. Furthermore, binding of substrate polypeptide chains to non-active subunits may determine the positions that become accessible to active sites. Our model concerning the trans-processing of the N terminus of Pre4 (Fig. 4) also suggests a minor importance of the P1 amino acid for cleavage at any active site. The Asn-Thr bond most probably cleaved by the catalytic center in Pup1 to yield mature Pre4 is not at all a typical site for trypsin-like specificity. In addition, Pre3, correlated with PGPH specificity, is obviously able to cut in a region of the Pre4 prosequence without acidic residues. Thus, the properties distinguishing the three different active sites of eukaryotic proteasomes remain to be analyzed in detail using longer polypeptides.

Active Subunits and Precursor Processing—The different phenotypes caused by neutralization of the different proteasomal active sites may imply a hierarchy in the importance of the three catalytic subunits. However, the same residues critical for proteolytic activity of the mature subunits are also involved in autocatalytic propeptide cleavage, a prerequisite for generation of the active site. For the archaeal Thermoplasma proteasome, the effects of active site mutations on processing of the β-subunit and on external peptide cleavage could be separated by deleting the prosequence, which was without severe effect on proteasome assembly (26). Mutation of Thr-81, Lys-β33, and other residues in this truncated version led to the same loss of peptidase activity as in a full-length version, which did not undergo maturation (25, 28). Thus, the effect of homologous mutations in eukaryotic β-type subunits on external peptide cleavage should be the same regardless of the presence or absence of the prosequence. But consequences on the overall proteasome function cannot solely be ascribed to mutational disturbance of a given active site since in eukaryotic proteasomes non-removed N-terminal subunit extensions might cause sterical problems for assembly and stability of the complex and even for the action of the non-mutated active sites. Although we have not yet analyzed processing of our pre3-T20A and pup1-T30A mutants, we must expect preservation of their prosequences. A drop of overall proteasome concentration due to assembly deficiencies is unlikely in the pre3 and pup1 mutant cells, since the peptidase activities depending on the non-mutated active subunits were not diminished. On the other hand, the cold sensitivity observed in the pup1-T20A strain (Table III) may be explained by decreased stability or inefficient assembly of the proteasome at lower temperatures. In the case of Pre2 with by far the longest prosequence, the Thr-76 → Ala mutation was lethal. Our data suggest that the impairment of Pre2 self-maturation rather than loss of chymotrypsin-like activity is responsible for this lethal effect. The growth defects induced by overexpression of the pre2-T76A mutant allele in a wild-type strain (not shown) point to an impediment of the assembly process by an excess of mutated subunit. As shown by the reduction of chymotrypsin-like activity in the shut-off experiment, incorporation of the Pre2-T76A variant can be enforced in the absence of wild-type Pre2 (Fig. 5). However, cells dependent on this mutated Pre2 obviously cannot survive for longer periods, in contrast to cells harboring Pre2 variants with Thr-76 exchanged to serine or with mutations at Lys-108. The pre2-K108A and the pre2-K108R mutants as well as the pre2-T76S strain were severely restricted in growth (Table III). Since only the former mutants lack chymotrypsin-like activity, the defective peptidase activity cannot be the only reason for poor growth. Immunological analysis of Pre2 species (Fig. 6) indeed revealed unprocessed pro-form accumulating in all three mutant strains. Pre2 precursor processing intermediates were found in both Lys-108 mutants, and no maturation of the precursor to wild-type molecular size was detectable in the pre2-K108A strain. Recent investigations on the effect of active site mutations in Pre2 on its processing (30) are consistent with our data. A Pre2 version lacking the prosequence was unable to suppress lethality of a PRE2 deletion, but expression of the propeptide in trans restored viability. When the prosequence was coexpressed with the mature Pre2 moiety mutated at Thr-76 or Lys-108, these mutations had only little effect on cell viability and could be directly correlated with loss of chymotrypsin-like activity. Furthermore, a detailed analysis with mutants expressing full-length Pre2 variants altered at the precursor processing site (pre2-T76S and pre2-G75A) revealed an accumulation of the unprocessed Pre2 subunit in crude extracts. Pre2 forms with only part of the prosequence cleaved off were detected in crude extracts as well as in purified mutant proteasomes. In contrast to Chen and Hochstrasser (30), who interpreted these forms as artificial degradation products, we explain these species as processing intermediates derived from cleavage by other catalytic centers, which accumulate due to impairment of the final autocatalytic processing step. In summary, we conclude that correct processing of Pre2 is needed to form stable proteasome complexes, probably due to the length of the propeptide. Once the necessity for removal of the Pre2 prosequence is circumvented by expressing it in trans, mutation of the “chymotrypsin-like” active site in Pre2 has only little effect on proteasomal functions, comparable to similar mutations in Pre3 and Pup1. Thus, a hierarchy among the roles of the three proteasomal active sites in substrate degradation still has to be proven.
The maturation event activating proteasomal threonine protease subunits most likely occurs autocatalytically (28, 30, 31), whereas N-terminal processing of non-active β-type subunits cannot be achieved by an intramolecular autocatalytic mechanism. Our data on the maturation of Pre4 in different active site mutants strongly suggest the involvement of neighboring active subunits. In particular, all activated sites in a 20 S proteasome precursor complex can participate in the length reduction of the Pre4 propeptide, and the most C-terminal cut remains to be investigated.

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