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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 nucleophilic 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 chymotrypsin-like activity. Double mutants harboring only trypsin-like or chymotrypsin-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.

The proteasome is a large multi-subunit proteinase complex found in the cytoplasm and nucleus of all eukaryotic cells examined so far. This "proteolytic organelle" fulfills vital cellular functions. As part of the ubiquitin-mediated protein degradation machinery, it is responsible not only for the elimination of misfolded proteins, including those derived from the lumen of the endoplasmic reticulum (1), it also controls a multitude of regulatory processes by removing unnecessary or even harmful metabolic enzymes and by balancing the levels of many regulatory proteins (for reviews see Refs. 2–4). Proteasomes exist as particles of 20 S and of 26 S. The 26 S complex of ≈2000 kDa is composed of the 20 S particle of ≈700 kDa as a proteolytic core unit and two regulatory 19 S caps that dock onto each side of the 20 S cylinder and confer ATP and ubiquitin dependence onto proteasomal protein degradation (2, 3, 5–7).

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 chymotrypsin-like, a trypsin-like, and a peptidyl-glutamyl peptide-hydrolyzing (PGPH)[1] 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 archaebacterial 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.

TABLE I
Generation of potential active site knock-out mutant alleles

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 *Ecl*136I/*Hin*dII)	GGTTGCTG*CGC*ATTAACCAT	New *Fsp*I site	p15-E4T34A
pre4-T42A		GACGGAA*GC*ACCTGTTACTA	Loss of *Rsa*I site	p15-E4T42A
pre3-T20A	p15-E3 (*PRE3*; 1.42-kbp *Nru*I/*Sna*BI)	*GCC*TCAATGTATGTATATAAGA	Loss of *Kpn*I site	p15-E3T20A
pup1-T30A	p15-P1[a] (*PUP1*; 1.77 kbp)	CCACGGGT*GCC*ACCATTGTA	Loss of *Kpn*I site	p15-P1T30A
pre2-T76A	p15-E2 (*PRE*2; 1.33 kbp, upstream end:	GTA*GC*ACCATGTGCGATCTTG	Loss of *Rsa*I site	p15-E2T76A
pre2-T76S	derived from exonuclease digestion,	GTA*GA*ACCATGTGCGATCTTG	Loss of *Rsa*I site	p15-E2T76S
pre2-K108A	downstream end: *Bam*HI)	AAC*GGC*CTTCACAGTTTGAGAAG	New *Hae*III site	p15-E2K108A
pre2-K108R		AAC*GCG*CTTCACAGTTTGAGAAG	New *Cfo*I site	p15-E2K108R

[a] See text for details.

amino group of Lys-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 archaebacterial and eukaryotic proteasomes was first implicated through the covalent binding of the natural, highly 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/Doa3, 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 archaebacterial 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 *Eco*RI/*Xho*I 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 appended by insertion of an *Xho*I-cut 0.65-kbp PCR fragment derived from genomic DNA with primers PUP1–5Xho (5′-CCGCTCGAGCTCCTCTTGGGAATCACT-3′) and PUP1-cenXho (5′-CTGAGACCACTCTTGGTTC-3′), yielding p16-P1. The 1.77-kbp insert was released by partial *Xho*I and *Bam*HI digest and cloned into pRS315.

Plasmid-dependent Putative Active Site Mutants (Plasmid Shuffle)—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 *pup1*Δ*::HIS3* null allele was constructed by cloning a p15-P1-derived 0.22-kbp *Xho*I/*Bcl*I fragment carrying 5′-flanking regions of *PUP1* together with a 1.77-kbp *Bam*HI fragment carrying the *HIS3* gene into *Sal*I/*Bam*HI-cut pUC18 and subsequently inserting 3′-flanking regions of *PUP1* on a 0.62-kbp *Afl*II(filled-in)/*Eco*RI fragment from p16-P1 between the *Sma*I and *Eco*RI sites yielding plasmid p18-P1Δ::HIS3. A *pup1* knock-out mutant was generated by one-step gene disruption (33) in strain WCG4a/α by transformation with the *pup1*Δ*::HIS3* fragment from p18-P1Δ::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/α, yielding the heterozygous *PRE2*/*pre2-T76A* diploid YWH22.

Overexpression of PRE2 Alleles; Galactose-inducible PRE2 and PRE5—For overexpression experiments the *PRE2* and *pre2-T76A* genes were cloned into the *LEU2*-marked high copy 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 *Nsp*I/*Bam*HI *PRE2* fragment from p15-E2 into *Sph*I/*Bam*HI-cut p15-GAL/Sph, a modified version of plasmid pRS315-GAL[2] with an additional *Sph*I site between the *Hin*dIII and *Sal*I sites. By this the *PRE2* translation-start ATG was positioned closely behind the *GAL1* promoter sequences in the resulting plasmid p15-GAL-E2. Plasmid p15-GAL-E5 was constructed analogously by inserting a 0.83-kbp *Nsp*I/*Sac*I *PRE5* fragment into *Sph*I/*Sac*I-cut p15-GAL/Sph. Strain YWH220 (*pre2*Δ*::HIS3* [p15-GAL-E2]) is a haploid descendant of a diploid *pre2*Δ*::HIS3*/*PRE2* strain transformed with p15-GAL-E2. Accordingly,

[2] P. Hieter, unpublished results.

TABLE II
Yeast strains used in this study

All strains are isogenic with WCG4a, WCG4α (22), or WCG4a/α, respectively.

Strain	Relevant genotype
WCG4a	*MAT**a** leu2–3,112 ura3 his3–11,15 Can^S GAL2*
WCG4α	*MATα leu2–3,112 ura3 his3-11,15 Can^S GAL2*
WCG4a/α	WCG4a X WCG4α; diploid
YWH10	*pup1Δ::HIS3* [p16-P1]
YWH11	*pup1Δ::HIS3* [p15-P1T30A]
YUS4	*pup1-T30A*
YWH30[a]	*pre3Δ::HIS3* [p16-E3]
YWH31	*pre3Δ::HIS3* [p15-E3T20A]
YUS1	*pre3-T20A*
YUS5	*pup1-T30A pre3-T20A*
YHI39–1/2[b]	*pre4Δ2::HIS3* [p16-E4]
YWH41	*pre4Δ2::HIS3* [p15-E4T34A]
YWH42	*pre4Δ2::HIS3* [p15-E4T42A]
YUS2	*pre4-T34A*
YUS3	*pre4-T42A*
YWH20[c]	*pre2Δ::HIS3* [p16-E2]
YWH215	*pre2Δ::HIS3* [p15-E2T76S]
YWH213	*pre2Δ::HIS3* [p15-E2K108A]
YWH214	*pre2Δ::HIS3* [p15-E2K108R]
YWH23	*pre2-K108A*
YWH24	*pre2-K108R*
YWH25	*pre2-T76S*
YWH26	*pre2-K108R pre3-T20A*
YWH22	*PRE2/pre2-T76A*
YWH220	*pre2Δ::HIS3* [p15-GAL-E2]
YWH221	*pre2-T76A* [p15-GAL-E2]
YWH222	*PRE2* [p15-GAL-E2]
YWH520[d]	*pre5Δ::HIS3* [p15-GAL-E5]
YWH200	*PRE2* [pRS425]
YWH201	*PRE2* [p25-E2]
YWH202	*PRE2* [p25-E2T76A]

[a] R. Gückel and W. Hilt, unpublished, disruption allele as in Ref. 24.
[b] M. Bernert and W. Hilt, unpublished, disruption allele as in Ref. 23.
[c] Unpublished, disruption allele as in Ref. 22.
[d] Unpublished, disruption allele as in Ref. 16.

strain YWH520 (*pre5Δ::HIS3* [p15-GAL-E5]) was derived by sporulation of a *pre5Δ::HIS3/PRE5* diploid harboring p15-GAL-E5. YWH22 (*PRE2/pre2-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 complete medium containing either glucose or galactose.

Measurements of Proteasomal Peptidase Activities and β-Galactosidase Activity—Conditions for colony overlay assays applied to indicate *in situ* proteasomal peptidase activity against the substrates *N*-Cbz-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 grown on solid medium was assayed as follows: cells grown as patches on YPD plates covered with sterile filter disks were permeabilized by soaking the filter in 3 ml of 50 mM Tris-HCl, pH 9.3, 1% toluene, 5% ethanol for 15 min. The dried filter was then covered with 10 ml of substrate solution made of 1% agarose, 50 mM Tris-HCl, pH 9.3, 0.5 mM each of EDTA and EGTA, and 100 μl of 50 mM *N*-Cbz-Ala-Arg-Arg-4-methoxy-β-naphthylamide in dimethyl sulfoxide. After incubation at 50 °C for 4–6 h released 4-methoxy-β-naphthylamide was converted to an azo dye as described (23). For measurements of peptidase activities in defined amounts of intact cells, about 1 A_{600} of cells per assay were washed with water and either permeabilized by adding chloroform to the moist cell pellet or, for determination of trypsin-like activity, by resuspending the cells in 100 μl of 50 mM Tris-HCl, pH 9.3, 1% toluene, 5% ethanol. After 15 min cells were collected and resuspended in 200 μl of enzyme assay solutions as described in Ref. 38 for fluorogenic peptide substrates and in Ref. 15 for *N*-Cbz-Gly-Gly-Leu-*p*-nitroanilide. Incubation of the reaction mixtures was done at 37 °C with vigorous shaking. After stopping the reactions the amount of released fluorophores or chromophores in the supernatants 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 analogously. 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 electroblotting have been described (15, 38). Fusion proteins between glutathione *S*-transferase and Pre2 or Pre4 were expressed in *Escherichia coli* from pGEX vectors (Pharmacia Biotech Inc.) and purified either from inclusion bodies according to standard protocols (32) or according to the protocol provided by the manufacturer. Polyclonal antibodies against the fusion proteins were raised in rabbits by the Eurogentech Co. For immunodetection by enhanced chemiluminescence (Amersham Corp.) following the manufacturer's protocol, the anti-Pre2 and anti-Pre4 antisera were used in 1:2000 and 1:5000 dilution, respectively.

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 archaebacterial 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 propeptide 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,[3] 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

[3] M. Bernert and W. Hilt, unpublished results.

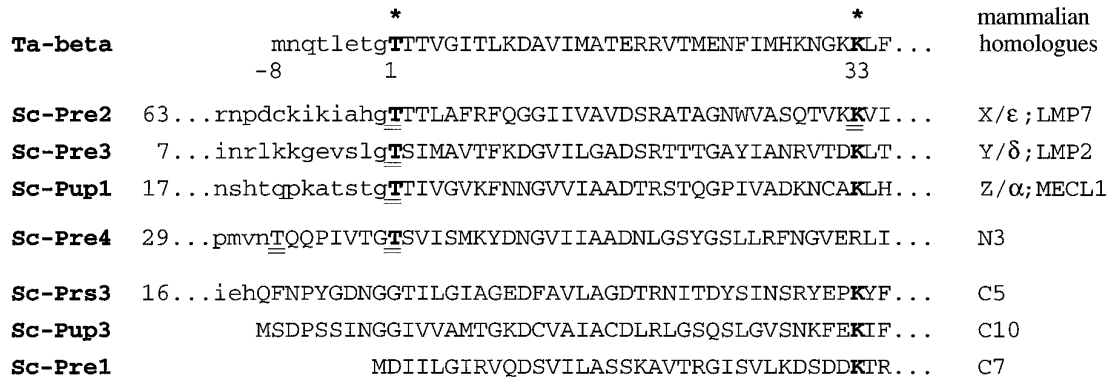

FIG. 1. **Sequence alignment of the N terminus from the *T. acidophilum* proteasome β-subunit (*Ta-beta*) with N-terminal regions from the seven *S. cerevisiae* (*Sc*) proteasomal β-type subunits.** The Ta-beta propeptide and fragments of yeast proteasome subunit propeptides are in *lowercase letters* and N-terminal parts corresponding to the matured proteins are shown in *uppercase letters*. (Only for Sc-Pre3 and Sc-Pre2 the processing site was experimentally determined, for the other yeast proteins data from homologous eukaryotic β-type subunits were taken.) *Numbering below* the Ta-beta sequence is related to the mature protein. *Numbers* to the *left* of the yeast sequences indicate omitted propeptide residues. *Stars* label the position of 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*.

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-β1 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 *pre4*Δ 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. 2*A*). 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 *pre4* mutants by gel filtration (not shown). In agreement with an almost unaffected 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 main peptidolytic activities of the proteasome nor in any proteolytic activity that is necessary for cell proliferation. The data imply also that N-terminal processing of Pre4 is unlikely to occur autocatalytically. To investigate whether the Thr/Ala exchanges in the two Pre4 mutant proteins would interfere with the processing event, we separated crude extracts of the mutant cells by SDS-polyacrylamide gel electrophoresis and visualized the Pre4 subunit by immunoblotting. The immunoreactive mutant Pre4 material (Fig. 3, *lanes 1* and *2*) migrated at the same position as did the mature subunit from wild-type cell extracts (Fig. 3, *lane 6*) or from purified wild-type 20 S proteasome (Fig. 3, *lane 7*). No unprocessed Pre4 precursor or processing intermediates were detectable. Since maturation of Pre4 was unaffected by the Thr-42 → Ala and Thr-34 → Ala mutations, an involvement of both residues in an autocatalytic processing reaction can be excluded. Furthermore, Thr-34 evidently does not serve as signal for precursor maturation by a protease splitting in *trans*.

Pre3 Bears the Catalytic Site for the PGPH Activity—Screening of mutagenized yeast cells for clones with defective proteasomal PGPH activity had yielded one *pre4* mutant (23) and several *pre3* mutants (24).[4] Since the mature Pre3 subunit contains an N-terminal threonine (40) and a lysine equivalent to Lys-β33 (Fig. 1), we considered that Pre3 is a threonine protease conferring 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. 2*B* 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-

[4] R. Gückel and W. Hilt, manuscript in preparation.

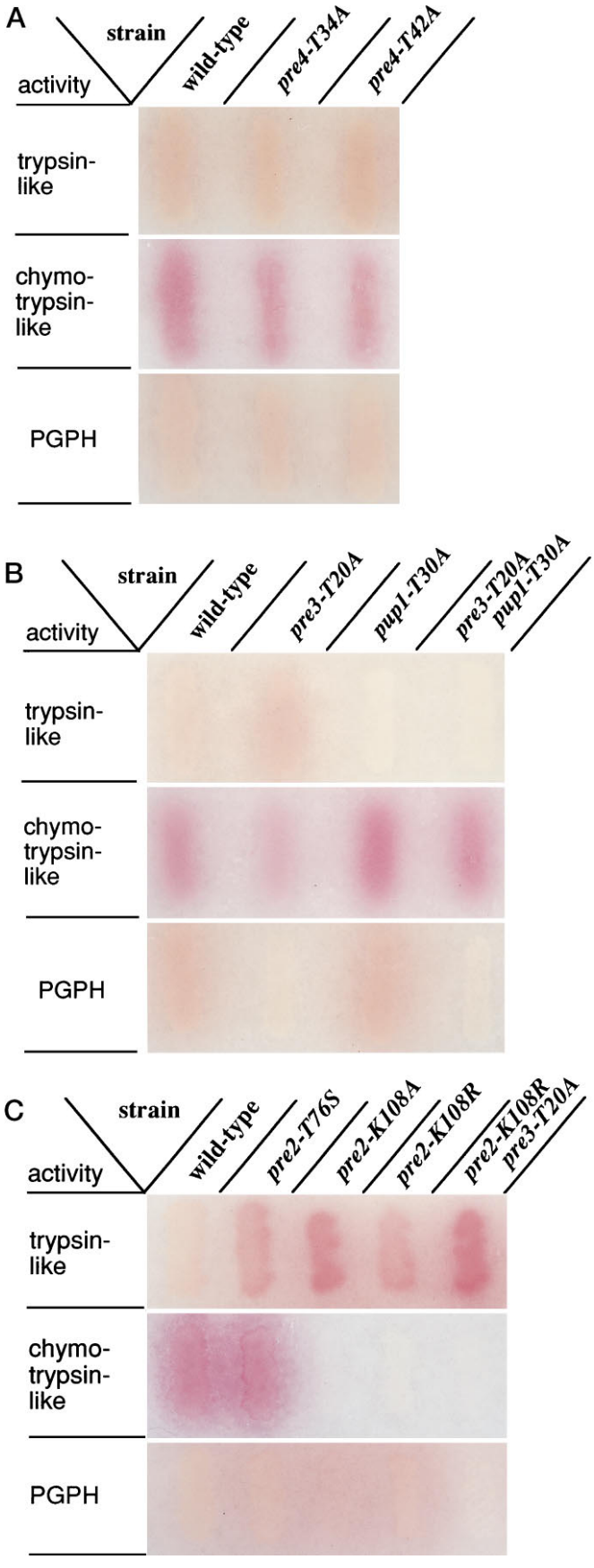

FIG. 2. ***In situ* assays for different proteasomal peptidase activities.** Patches of yeast cells chromosomally harboring the proteasomal mutant alleles as indicated were grown in triplicate on filters, permeabilized, and covered with gels containing different peptide substrates to assay trypsin-like (*N*-Cbz-Ala-Arg-Arg-4-methoxy-β-naphthylamide), chymotrypsin-like (*N*-Cbz-Gly-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*).

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. 2*B* 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 peptidase activity (Fig. 2*B* 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

TABLE III

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	+++++	+++++	+++++
YUS2	*pre4-T34A*	80	113	110	+++++	+++++	+++++
YUS3	*pre4-T42A*	93	97	100	+++++	+++++	+++++
YUS1	*pre3-T20A*	7	164	89	+++++	+++++	+++++
YUS4	*pup1-T30A*	85	15	86	++++	++	++++
YUS5	*pup1-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	+++	+++	+

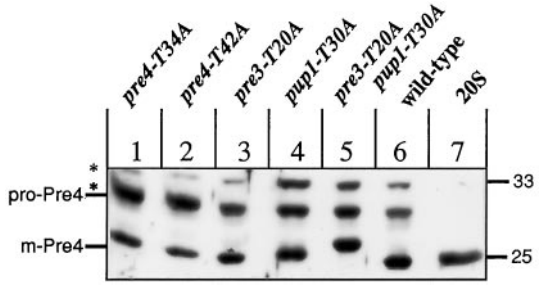

FIG. 3. **Anti-Pre4 immunoblot analysis of potential active site mutants in Pre4, Pre3, or/and Pup1.** Crude extracts of cells with the genotypes as indicated were prepared from stationary cultures and separated on an 18% polyacrylamide SDS-gel prior to electroblotting and detection of immunoreactive Pre4 protein. Molecular mass standards (in kDa) are presented on the *right*. The positions of non-processed (*pro-Pre4*) and completely processed (*m-Pre4*) Pre4 species are marked on the *left*. Bands derived from unspecific reaction of the anti-Pre4 immunoserum with unknown proteins are labeled with *asterisks*. Note that the intense band of cross-reacting material covers up minor amounts of pro-Pre4 only seen in *lanes 4* and *5* after shorter exposure. *20 S*, purified yeast 20 S proteasome reference sample.

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 form 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 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).[5] 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 *pre2*Δ 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 *pre2*Δ 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

[5] W. Heinemeyer, unpublished results.

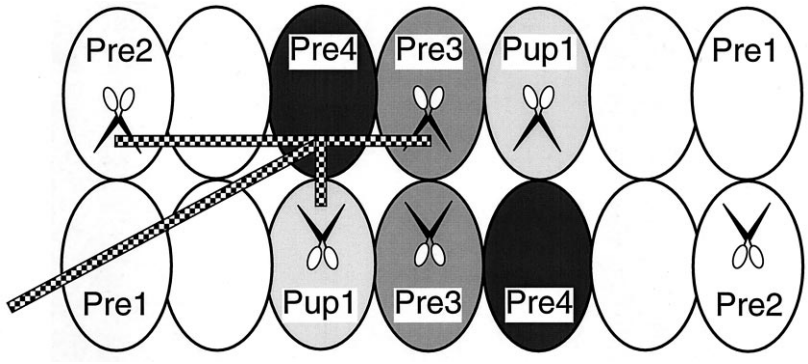

FIG. 4. **Arrangement of β-type subunits in the yeast 20 S proteasome and Pre4 precursor processing in wild-type and active site mutants.** The two central proteasomal β-type subunit rings are presented in linearized form. (The subunit topography is based on latest data (58) and differs slightly from that suggested for the mammalian proteasome (45) in that the positions of Pre1 and Pre2 are exchanged.) Scissors label proteolytically active centers. The propeptide of one Pre4 subunit is represented by the *longest bar*. Cleavage of this propeptide at the catalytic sites located in Pre2, Pre3, or Pup1 is indicated by the *shortened bars* directed toward these subunits. The *vertical bar* corresponds to the N terminus of completely processed Pre4, derived by the Pup1 activity. The *bars* directed toward Pre3 or Pre2 correspond to incompletely processed Pre4 N termini found in *pup1* single or *pup1 pre3* double active site mutants, respectively.

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 *pre2*Δ control strain as well as in a *pre5*Δ control strain (deleted for an α-type proteasomal 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 *pre2*Δ and *pre5*Δ 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 precursor 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 *pre2*Δ 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. 2*C* 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 archaebacterial 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.[5]

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. 2*C* 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 unimpaired 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. 2*C* 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. 2*C* 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

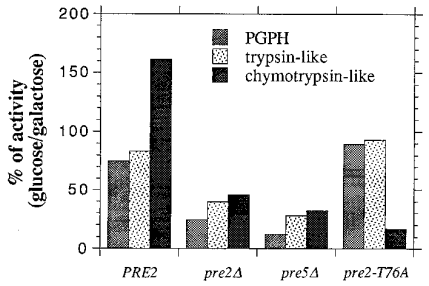

FIG. 5. **Thr-76 → Ala-mutated Pre2 is incorporated into proteasomes leading to loss of chymotrypsin-like peptidase activity.** Strains chromosomally harboring the alleles as indicated were transformed with plasmids expressing wild-type *PRE2* (or *PRE5* in the case of the *pre5*Δ strain) under control of the *GAL1* promoter. *In situ* peptidase activities (substrates as outlined in legend to Fig. 2) were determined after culturing the strains for 15 h at 30 °C in derepressing galactose or in repressing glucose medium. The percentage of each activity in cells grown in glucose relative to the activities in cells grown in galactose was calculated. The mean values given were derived from measurement of each two parallel cultures.

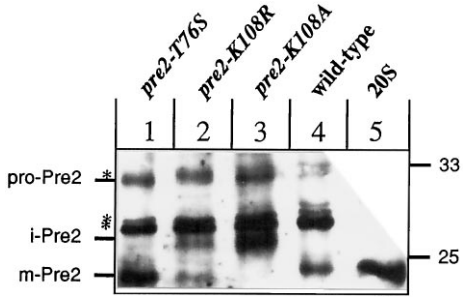

FIG. 6. **Anti-Pre2 immunoblot analysis of active site mutants in Pre2.** Crude extracts of cells with the genotypes as indicated were prepared from stationary cultures, separated on an 18% polyacrylamide SDS-gel, and subjected to immunoblot analysis using an anti-Pre2 antiserum. Molecular mass standards (in kDa) are presented on the *right*. The positions of non-processed (*pro-Pre2*), incompletely processed (*i-Pre2*), and matured Pre2 (*m-Pre2*) are marked on the *left*. Bands derived from unspecific reactions of the anti-Pre2 immunoserum with unknown proteins are labeled with *asterisks*. *20 S*, purified yeast 20 S proteasome reference sample.

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 β-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* β-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 δ/Y, α/Z, and ε/X/MB1 by the γ-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 findings were in part controversial and not reflected by the cleavage patterns of longer substrate proteins (50, 52, 54). However, since mice lacking LMP2 or LMP7 indeed have defects in antigen presentation (55, 56), the subunit exchanges in immunoproteasomes are most likely of relevance for their functional adaptation. This is supported by our finding that the yeast homologues of the interchangeable mammalian subunits are proteolytically active.

The mammalian β-type subunit N3 was a questionable candidate concerning proteolytic activity (25) because it contains an arginine residue at the position homologous to Lys-β33 and starts with an N-terminal threonine located upstream of the threonine in the Thr-β1 position. Our mutational analysis of both threonines in the yeast homologue of N3, Pre4, largely rules out the possibility that members of this subunit branch represent active threonine proteases with a modified catalytic center. Orlowski *et al.* (57) found two additional peptidase activities in rat 20 S proteasomes that differed from the three major specificities in their response to certain inhibitors. As long as these new activities have not been attributed to the three threonine protease subunits, additional catalytic centers cannot completely be excluded. Other proteasomal active sites would have to use, however, a catalytic mechanism different from the threonine protease subunits, since members of the remaining three β-type subunit branches represented by the yeast Pre1, Pup3, and Prs3 proteins are devoid of Thr-1.

While this manuscript was under review the structure of the yeast 20 S proteasome determined by x-ray crystallography was published (58). The data agree with our finding that three kinds of threonine protease subunits exist in the enzyme complex. A co-crystallized peptide aldehyde inhibitor was found covalently bound to the terminal threonines of Pre3, Pup1, and 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 α-helices that are present in all 7 β-type subunits and that together form an anulus surrounding the inner surface of each β-ring. This hypothesis is based on the finding that the N termini of both partially processed, non-active subunit neighbors Pre4 and Prs3 were situated at one of these α-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 β-anulus not to be involved in subunit processing, and we also doubt a proteolytic function of this β-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 lactacystin to the catalytic center of Pre2. In mammalian proteasomes lactacystin inhibited both the chymotrypsin-like and the trypsin-like activities by modifying one subunit, the Pre2 homologue X/MB1 (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. 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-β1, 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-T30A* 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 is exerted across the β-rings by the nearest active neighbor, Pup1, without the need for preceding cleavage by Pre2 or Pre3 (Figs. 3 and 4). Interestingly, the situation of Pre4 processing is reminiscent of the intermediate precursor processing products found for active site mutant forms of the mammalian LMP2 subunit, a homologue of Pre3 (31). In these LMP2 variants, cleavage in the propeptide was found to occur 8–10 residues upstream from the Thr-1 position. From these data a model of an ordered two-step processing mechanism was deduced (31) which also favors the involvement of other, already activated threonine protease subunits in an initial length reduction preceding the final autocatalytic activation step. This was substantiated by the prevention of *both* processing steps by a proteasome-specific inhibitor. Further support comes from the Pre2 processing intermediate observed in our Pre2 active site mutants (Fig. 6). In agreement with the localization of Pre2 in the proteasome, this processing intermediate apparently has a longer N-terminal extension of at least 15 amino acids, which roughly matches the minimal distance to the next active threonine protease subunits, Pup1 and Pre3. However, a "pre"-processing in *trans*, as suggested by Schmidtke *et al.* (31), is obviously not essential for the final autocatalytic maturation step to occur. Our viable *pre3 pup1* and *pre3 pre2* active site double mutants could not gain chymotrypsin-like and trypsin-like activity, respectively, if an initial length reduction of Pre2 or Pup1 were necessary for their final self-activation. Furthermore, Pre2 maturation was found to occur normally in the *pre3 pup1* mutant (not shown). Thus, cleavage of β-type subunit precursors by other active site-carrying subunits might be a "side reaction" only seen in non-active or in mutationally inactivated subunits. Whether the efficiency of self-maturation of active site subunits is improved by a length reduction step remains to be investigated.

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ENZYMOLOGY:

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

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