Proteasomes are highly complex proteases responsible for selective protein degradation in the eukaryotic cell. 26 S proteasomes consist of two regulatory 19 S cap complexes and the 20 S proteasome, which acts as the proteolytic core module. We isolated six mutants of the yeast Saccharomyces cerevisiae containing mutations in the 20 S proteasome β-type subunit Pre3. Three mutations (pre3–2, pre3–3, and pre3–5) which reside at the active site cleft of the Pre3 subunit solely caused reduction of the proteasomal peptidylglutamyl peptide-hydrolyzing activity but did not lead to detectable defects in protein degradation nor to any other phenotype. However, the pre3–2 mutation strengthened phenotypes induced by other 20 S proteasomal mutations, indicating that the peptidylglutamyl peptide-hydrolyzing activity has to fulfill some rescue functions. The other three mutations (pre3–1, pre3–4, and pre3–6) are located at diverse sites of the Pre3 protein and caused multiple defects in proteasomal peptide cleaving activities. pre3–1 and pre3–6 mutants exhibited significant defects in proteasomal protein degradation; they accumulated ubiquitinated proteins and stabilized defined substrate proteins as, e.g. fructose-1,6-bisphosphatase. In addition, pre3–1 and pre3–6 mutant cells exhibited pleiotropic phenotypes as temperature sensitivity and cell cycle-related effects.

Proteasomes are large proteinase complexes operating in the cytoplasm and the nucleus of the eukaryotic cell. Two complexes are found: (i) the cylindrically shaped 20 S proteasome (molecular mass ~700 kDa) and (ii) the larger 26 S proteasome (molecular mass ~1700 kDa), which is composed of the 20 S proteasome as the proteolytic core and two additional 19 S cap complexes attached at both ends of the 20 S cylinder. Proteasomes are the major tool for selective protein degradation in the cytoplasm and nucleus of the eukaryotic cell (2–4). 26 S proteasomes degrade ubiquitinated proteins in an ATP-dependent reaction, whereas 20 S proteasomes are not able to do so. It is thought that the 19 S cap complexes of the 26 S proteasome, which consist of ATPases associated with various cellular activities and non-ATPase subunits are responsible for recognition, unfolding and transport of a substrate protein to the proteolytically active 20 S core. Using artificial peptide substrates, the 20 S proteasome exhibits at least three different proteolytic activities characterized by the type of amino acid where cleaving occurs: the chymotrypsin-like activity, the peptidylglutamyl peptide-hydrolyzing (PGPH)1 activity and the trypsin-like activity clipping at the C terminus of hydrophobic, acidic, and basic amino acids, respectively (5–8).

The cylindrically shaped 20 S proteasome consists of a stack of four rings, each containing seven subunits (4). In eukaryotes the outer rings are built up of seven different α-subunits, and the inner rings are composed of seven different β-subunits, each. X-ray structure analysis of the ancestor 20 S proteasome from the prokaryote Thermoplasma acidophilum, which is composed of only one type of α- and β-type subunit revealed the particle to be a hollow cylinder (9). The channel spanning the center of the complex is subdivided in three cavities. Both outer cavities are formed by the interfaces of the α and β rings, whereas the central cavity, which contains the proteolytically active sites, is build up only from β rings (9). X-ray structure as well as mutational analysis uncovered the N-terminal threonine of the β-type subunit to be essential for the proteolytic activity of the Thermoplasma proteasome (9, 10). Access to the active compartment is controlled by four narrow gates, and proteins have to be unfolded before degradation. This general design of the 20 S proteasome complex has recently been confirmed for eukaryotes by x-ray structure analysis of the yeast 20 S proteasome at a 2.4-Å resolution (11). This study in addition resolved the subunit arrangement of the eukaryotic 20 S proteasome, as well as structural details of individual subunits of the eukaryotic proteasome. X-ray structure in combination with lactacystin binding analysis determined the active sites of the yeast proteasome to locate at the β-type subunits Pre2, Pre3, and Pup1 (11). Utilizing site directed mutagenesis of the essential N-terminal threonine residues of these subunits, the individual peptide cleaving activities could be assigned to one of these active subunits, each (12, 13); Pre2 confers the chymotrypsin-like activity, Pre3 the PGPH activity, and Pup1 the trypsin-like activity.

Proteasome-mediated degradation is linked to many different pathways of the cell. Proteasomes are implicated in stress response. They remove abnormal proteins generated by heat stress or exposure of cells to amino acid analogues (6, 14–16). Recent work demonstrated that proteasomes even degrade ab-

1 The abbreviations used are: PGPH, peptidylglutamyl peptide-hydrolyzing; Cbz, carbobenzoxy1; 5FOA, 5-fluouracil acid; β-gal, β-galactosidase; FBPase, fructose-1,6-bisphosphatase; CDK, cyclin-dependent kinase complex; bp, base pair(s); CM, complete medium; PCR, polymerase chain reaction; βNA, β-naphthylamide.
normal luminal proteins of the endoplasmic reticulum, which have been transported to the cytoplasm prior to degradation (17, 18). Proteasome-dependent degradation of one or several defined substrate proteins has been found to constitute a significant step in various regulatory pathways of the cell. Proteasomes are involved in adaptation of metabolism by degrading enzymes as fructose-1,6-bisphosphatase (19–21) or ornithine decarboxylase (22) as well as transcriptional regulators like Gcn4, which controls expression of metabolic enzymes (23). Proteasomes are also linked to cell differentiation as had been shown for the proteolytically unstable yeast MATa2 transcriptional repressor protein (24, 25).

Most importantly, proteasomes play vital functions in the cell division cycle (for reviews, see Refs. 26–28). In yeast, the central cdc2/Cdc28 kinase is cell cycle phase specifically activated by association with certain cyclin proteins. Such cyclin-dependent kinase complexes (CDKs) are inactivated by proteasome-mediated degradation of the respective cyclin proteins by this terminating distinct phases of the cell cycle (29, 30). Two different pathways act in recruiting cyclins for proteasomal destruction. G1 cyclins are tagged via Cdc34 complexed with Skp1, Cdc53, and the F-box protein Cdc2 (SCF pathway) (for review, see Ref. 31). In contrast, the anaphase-promoting complex mediates B-type cyclin destruction in late mitosis (for review, see Refs. 28 and 31). Proteasomal destruction is also needed for removal of CDK inhibitor proteins as, e.g., the Sic1 protein (32). In contrast to cyclin destruction here proteolytic degradation results in activation of the respective CDK complex. Moreover, proteasomes degrade other cell cycle controlling proteins, which are not associated with cdc2/CDC28 kinase; anaphase-promoting complex mediated degradation of Pds1 (33), or Cut2 in Schizosaccharomyces pombe (34) and Ase1 (35) define essential transitions in mitosis.

In agreement with the fact that the β-type subunits of the 20 S proteasome contain the proteolytically active sites previous screens for yeast cells with defects in 20 S proteasomal activities yielded only mutants harboring mutations in subunits of this type: Mutations residing in subunits Pre1 and Pre2 caused defects in chymotrypsin like activity (6, 14), whereas mutants harboring mutations in the proteasomal subunits Pre3 and Pre4 showed defective PGPH activity (15, 36). Interestingly, mutants with impaired chymotrypsin like activity (pre1 and pre2) exhibited strong reduction in degradation of proteasomal substrates (6, 20, 24, 37, 38) whereas pre4–1 mutants which lack the PGPH activity did not show any decrease in protein degrading activity (15) W. Hilt unpublished data.

Here, we present analysis of six pre3 mutant alleles; three mutations (pre3–2, pre3–3, pre3–5) residing in the substrate binding cleft of the Pre3 subunit exclusively caused defects in the PGPH activity of the complex. Interestingly, these mutations led to no detectable deficiency in proteasomal protein degradation. In addition, no other significant phenotype was found for these class of mutants. Three other mutations residing at distinct positions of the Pre3 subunit led to reduction of PGPH activity but also impaired other peptide cleaving activities. Two of these mutations, pre3–1 and pre3–6, led to strong defects in proteasomal proteolysis. These mutations in addition caused other significant phenotypes; mutants exhibited slow growth, sensitivity to elevated temperatures, impaired growth due to ectopical expression of cyclins, and other cell cycle-related effects.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA restriction and modifying enzymes and the HindIII linker were obtained from Boehringer (Mannheim, Germany). Peptide substrates were from Bachem (Basel, Switzerland). AutoRead™ sequencing- and FluoroPrime labeling kit were from Amersham Pharmacia Biotech (Freiburg, Germany). S-POA was from Toronto Research Chem Inc. (North York, Canada).

**Proteasome pre3 Mutants**

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TABLE I

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<tr>
<th>Strain</th>
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<td>(14)</td>
</tr>
<tr>
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<td>MAT pre1-1 pre3-2 his3-11,15 leu2-3,112 ura3 Can8 Gal+</td>
<td>This work</td>
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a This strain is isogenic with WCG4a.

b These strains are isogenic with WCG4a.

Cloning of the pre3 Mutant Alleles—Standard molecular biological methods were used (40). The pre3 mutant alleles were cloned by the gap repair method (44) or utilizing PCR amplification. For cloning by gap repair, the PRE3 3’ flanking region (250-bp XmnI/NruI fragment) and the 5’ flanking region (248-bp NruI/EcoRI fragment) were joined by a EcoRI linker and cloned into the shuttle vector pRS316 (CEN6 LEU2) (45). The resulting plasmid was linearized with EcoRI, and pre3 mutant strains were transformed. Uracil auxotrophic strains obtained were checked for the presence of repaired plasmids by plasmid rescue and restriction analysis. This approach was only successful in the case of strain C19 (pre3-3) allele. The other pre3 mutant alleles were therefore cloned by PCR amplification. The 1.4-kilobase pair NruI/SnaBI fragments containing the pre3 mutant loci were amplified using chromosomal DNA from strains C6, C13, FD80, and FD207 as templates and 5’-AAGAGGATCCGATACGTAGATACAGTC ACA and 5’-AAAGGATCCGATACGTAGATACAGTC ACA as primers. Both primers contained a BamHI site at their 5’ end, which was used for subcloning of the PCR fragments to the shuttle vector pRS315 (CEN6 LEU2) (45) yielding plasmids pRG21-pRG26.

Construction of Strains—Standard yeast genetic and microbiological techniques (41) were used. Strains C6, C13, C19, FD80, and FD207 were generated by ethylmethanesulfonate mutagenesis of strain plasmids pRG21-pRG26. A diploid pre3 knock-out mutant was generated as follows: A pRS316 based plasmid harboring a 1.4-kilobase NruI/SnaBI PRE3 fragment was gapped by HpaI digestion removing the complete PRE3 coding region and 6 bp from the 3’ region as well as 47 bp from the 5’ region. A 1.1-kilobase pair HindIII URA3 fragment excised from yEP24 was inserted using HindIII linker ligation. The deletion construct was excised as a BamHIXhol fragment. The diploid strain WCG4a was transformed with the linear fragment and uracil prototrophic strains were selected. To check correct deletion of PRE3 in strain YRG8, cells were sporulated and segregation of auxotrophic markers was determined. Spores derived from YRG8 tetrads showed a 2:0 segregation pattern (2 viable [ura3 PRE3]; 2 inviable [ura3::URA3]). Correct integration was further confirmed by PCR. pre3 single mutant strains (YRG11-YRG16) isogenic with WCG4a were generated by a one-step gene replacement and checked for correct recombination by PCR. Strain YHI54/3211 is a spore clone derived from a cross of YRG12 against YHI29/1 (Table 1).

Sequence of Mutant Alleles—The pre3 mutant alleles were sequenced by the dideoxy chain termination method using pRG21-pRG26 plasmids as templates. Double strand sequencing strategy was used as outlined in Ref. 36. Each allele was sequenced from the NruI site located in the PRE3 5’ region up to 70 bp downstream from the PRE3 structural gene. Sequencing was performed on an A.L.F. automatic DNA-sequencer (Amersham Pharmacia Biotech) using the AutoRead™ sequencing kit and the FluoroPrime™ labeling kit.

RESULTS

Isolation of pre3 Mutants—A series of mutants containing mutations in the 20 S proteasome β-type subunit Pre3 have been isolated. Three pre3 mutants (pre3-1 to pre3-3) had been found by screening ethylmethanesulfonate-mutagenized yeast cells for colonies with reduced PGPH activity of the 20 S proteasome (36). An independent mutant screen searching for cells that were defective for the trypsin-like and/or the PGPH activity of the proteasome yielded two additional mutants allelic with PRE3, which were called pre3-4 and pre3-5. McCusker and Haber (46) had performed a screen for yeast mutants that showed resistance to a minimal concentration of cycloheximide and in addition exhibited temperature sensitivity. One of these mutants, cr121, uncovered to be allelic with PRE3 (16), which in this work is referred to as pre3-6.

Compared with wild type cells, all these pre3 mutants, pre3-1 through pre3-6, exhibit reduced PGPH activity when using Cbz-Leu-Leu-Glu-βNA as a substrate. Three of these mutants, pre3-1, pre3-4, and pre3-6, additionally show defects in the trypsin-like activity of the 20 S proteasome. The pre3-6 mutant additionally is impaired in chymotrypsin like activity of the proteasome (data not shown; compare Fig. 1).

Isolation of pre3 Mutant Alleles—The pre3-3 allele was rescued by the gap repair method. pre3-3 mutant cells were transformed with a linear DNA fragment containing 3’ and 5’ homologous DNA sequences of the PRE3 promoter and terminator region separated by the backbone of the yeast shuttle vector pRS316 (URA3 CEN6). Plasmids repaired by homologous recombination were isolated from plasmid-dependent uracil prototrophic cells. Interestingly, in an attempt to rescue the other pre3 alleles, the gap repair method failed. We suggest this result to be due to reduced rates of homologous recombination found at regions adjacent to centromeres (49) as is the PRE3 locus. Therefore, the other pre3 alleles were isolated by PCR using primers which matched at 5’ and 3’ regions of the PRE3 locus yielding a 1.4-kilobase DNA fragment containing the sequence coding for the respective pre3 mutant allele, and flanking regions containing 341 base pairs of the PRE3 promoter and 327 base pairs of the terminator sequence. The fragments were subcloned to the yeast centromere vector pRS315 (LEU2 CEN6) yielding plasmids pRG21 through pRG26 and sequenced.

Generation of Isogenic pre3 Mutants—The diploid yeast strain (yRG8) which contained a heterozygous chromosomal preΔ::URA3 deletion was transformed with plasmids pRG21-pRG26 containing the pre3 mutant alleles. Cells were sporulated and tetrads dissected. Colonies derived from the...

Additional evidence for a failure in proteasomal protein degradation of pre3–1 mutants came from measuring β-galactosidase activity in cells expressing plasmid encoded proteolytically unstable versions of β-galactosidase. Such β-gal derivatives, (the so called N-end rule substrates; Ref. 50) had been proven to be degraded via the ubiquitin-proteasome pathway (37, 38). As compared with the wild type strain, pre3–1 cells (YRG11) expressing plasmid-encoded short-lived R-β-gal or Ub-P-β-gal exhibited enhanced β-galactosidase activity, indicating reduced turnover of this substrate protein in pre3–1 mutant cells (Fig. 3B). No enhanced activity levels were found in pre3–2, pre3–3, pre3–4, and pre3–5 mutant strains (Fig. 3B). Interestingly the pre3–6 mutant strain YRG16 did not tolerate R-β-gal or Ub-P-β-gal encoding plasmids. When transformed with R-β-gal or Ub-P-β-gal containing plasmids strain YRG16 exhibited extremely poor transformation rates. The few transformants obtained in addition showed very slow growth and could not be used to determine β-galactosidase activity in YRG16 cells.

A general defect in proteasomal proteolysis was substantiated in pre3–1 and pre3–6 mutants by measuring catabolite degradation of FBPase, a defined in vivo substrate of the ubiquitin-proteasome pathway (19–21). For both mutants, pre3–1 and pre3–6 strong proteolytic stabilization of FBPase was detected (Fig. 3C). In contrast, FBPase was degraded at wild type rates in pre3–2 mutant cells (Fig. 3C). In addition, utilizing Western blot-based pulse-chase experiments, no stabilization of FBPase was detected in pre3–3, pre3–4, and pre3–5 mutant cells (data not shown).

pre3–1 and pre3–6 Mutants Exhibit Growth Defects and Cell Cycle-related Defects—Overexpression of certain cyclins acting in different phases of the cell cycle is not tolerated in pre3–1 and pre3–6 mutant cells. Ectopic expression of Gal promoter controlled plasmid encoded G1 cyclins CLN2 (Fig. 4A), CLN1 and CLN3 (data not shown) in strains YRG11 (pre3–1) and YRG16 (pre3–6) nearly completely abolished cell growth. Re-
duced growth was also found when overexpressing the S/G2 cyclin CLB5 (Fig. 4B) and to a minor extent the mitotic cyclin CLB2 in pre3–6 mutant cells (Fig. 4C). In pre3–1 mutant cells, CLB5 overexpression only slightly impaired cell growth (Fig. 4B), whereas no significant growth defect was observed when overexpressing CLB2 in pre3–1 cells (YRG11) (Fig. 4C). No such sensitivity to ectopically expressed cyclins were found in the other pre3 mutant strains: when overexpressing any cyclin in pre3–2, pre3–3, pre3–4, and pre3–5 strains, no additional reduction in cell growth as compared with wild type cells occurred (Fig. 4 and data not shown).

pre3–2, pre3–3, pre3–4, and pre3–5 mutant cells grow at normal wild type rates at 30 °C (μ = 0.22 h⁻¹) and 37 °C (Fig. 5A). Previously, we had found that a mutation residing in the Pre3 neighbor subunit Pre4 which also exclusively causes reduction of the proteasomal PGPH activity could enhance proteasomal defects induced by the pre1–1 mutation, which resides in another β-type subunit and leads to defective chymotrypsin-like activity of the proteasome (15). An identical effect was found for pre3–2. This mutation when combined with the pre1–1 mutation significantly strengthened pre1–1 induced temperature sensitivity (Fig. 5B). In contrast to the other four pre3 mutant strains, pre3–1 and pre3–6 cells grow at reduced rates at 30 °C (pre3–1: μ = 0.11 h⁻¹; pre3–6: μ = 0.08 h⁻¹). As typically found for mutants defective in proteasomal degradation (6, 14–16, 52), both mutant strains, YRG11 (pre3–1) and YRG16 (pre3–6), show temperature sensitivity at 37 °C (Fig. 5A). When grown at 30 °C, YRG11 and YRG16 cells exhibited significantly reduced amounts of cells containing 1n DNA indicating premature entry into S-phase or prolonged transition through G2/M phase of the cell cycle (Fig. 6). After 2 h of incubation at 37 °C wild type and pre3–2 mutant cells show
enhanced amounts of G1 cells (Fig. 6). This behavior is assigned to the transient G1 arrest that is known to occur after heat shock (55). As expected this arrest is resolved during further incubation (5 h). The same effect may also be responsible for increase of 1n DNA and decrease of 2n DNA amounts in pre3–1 and pre3–6 cells after 2 h of incubation at 37 °C. However, pre3–1 and pre3–6 cells, which stop growth at this elevated temperature, remain with this 1n/2n DNA ratio even after 5 h of incubation (Fig. 6). Data clearly show that pre3–1 and pre3–6 mutant cells do not arrest in a defined phase of the cell cycle and that temperature sensitivity of pre3–1 and pre3–6 mutants is a cell cycle-independent event.

Mutant strains YRG11 (pre3–1) and YRG12 (pre3–6) produce certain amounts of missshapen cells. Cells emerging a prolonged tubular bud or exhibiting dumbbell shape appear in YPD cultures of these strains (pre3–6: 4–6%; pre3–1: 2–3%; wild type and pre3–2 < 0.4% in 24-h YPD) (Fig. 7). Amounts are significantly enhanced after growth to stationary phase (pre3–6: 12–15% pre3–1: 4–8% after 3 days of growth in YPD).

**DISCUSSION**

We have isolated mutants carrying mutations in the 20 S proteasome subunit Pre3. Concerning their localization in the Pre3 structure as well as their influence on peptide cleaving activities of the 20 S proteasome, the isolated pre3 mutations can be subdivided in two groups. The first group of mutations (pre3–2, pre3–3, and pre3–5) residing near the active site cleft of the Pre3 subunit solely cause reduction of the PGPH activity of the 20 S proteasome. The second group of mutations (pre3–1, pre3–4, and pre3–6) locate at diverse sites of the Pre3 structure and lead to multiple defects in several peptide cleaving activities of the proteasome complex. Two of these mutations, pre3–1 and pre3–6, result in strongly impaired protein degradation via the proteasome pathway and cause other pleiotropic phenotypes. In contrast, pre3–4 mutants, which are only slightly reduced in trypsin-like and PGPH activity as well as all class I mutants, do not show any deficiency in proteasomal protein degradation or any other cellular phenotype.

X-ray structure and mutational analysis uncovered the Pre3 protein to carry an active site, which is thought to be responsible for the PGPH activity of the 20 S proteasome (11–13). This finding is confirmed by the fact that pre3–2, pre3–3, and pre3–5 mutations lead to sole reduction of the PGPH activity of the proteasome. All three mutants contain a mutation of glycine 47. This residue is completely conserved in all β-type subunits analyzed so far and is part of the highly conserved so called GD-box spanning from Gly-47 to Asp-51. The strong conservation indicates that this sequence motif constitutes a structure essential for the function of the proteasome. X-ray structure data suggested that Gly-47 may essentially contribute to substrate cleavage. A fully occupied solvent molecule, which is thought to serve as the nucleophile attacking substrate molecules during hydrolysis, has been found in close vicinity to Gly-G47. In addition, x-ray structure analysis of T. acidophilum, and S. cerevisiae 20 S proteasomes treated with the inhibitor acetyl-Leu-Leu-norleucinal detected a hydrogen bond between Gly-47 and the inhibitor molecule. Moreover, Gly-47 locates to the center of a small loop between the S4 β-sheet and the H1 α-helix (11). The adjacent residues Arg-45 and Ala-49 form the bottom of the binding cleft of the Pre3 subunit. Moreover, residue Arg-45 is thought to comprise the site responsible for specific binding of substrates containing an acidic amino acid at the cleavage site (11). Exchange of Gly-47 against larger amino acids may disturb coupling of the nucleophilic water molecule to the binding cleft. In addition, such new residue may reach into the binding cleft and sterically hinder substrate...
binding. One may alternatively imagine that exchange of Gly-47 against other residues may result in distortion of the sharp bend formed by Gly-47. By this, disarrangement of Arg-45 and Ala-49 may occur, which finally may lead to a strong deficiency in substrate binding. 

Data clearly indicate that the pre3–2, pre3–3, and pre3–5 mutations lead to a specific effect which is limited to the Pre3 subunit of the 20 S proteasome. Interestingly, though almost completely lacking the proteasomal PGPH activity, no detectable defect on protein degradation nor any other additional cellular phenotype was found in these mutants. Identical results (loss of PGPH activity but no detectable defect in proteasomal protein degradation) had previously been obtained for pre3T1A mutants containing an exchange of the essential N-terminal threonine (13) as well as pre4–1 mutants lacking 15 amino acids at the C terminus of the Pre3 β-ring neighbor Pre4 (15). It had been discussed that mutants, although completely deficient in cleaving the artificial peptide substrate Cbz-Leu-Leu-Glu-bNA, may exhibit some residual activity for hydrolysis of peptide bonds within natural substrate proteins (15). This leakage model may now be excluded due to the fact that three structurally quite different mutations such as pre4–1, pre3T1A, and pre3G47D(S) lead to the same phenotypic effect. The data strongly indicate that loss of the PGPH activity does indeed not result in any deficiency in protein degradation via the proteasome. In case proteasomes lacking the PGPH activity were indeed fully active, the question arises why this activity has not been lost during evolution. Interestingly, pre4–1 (15) or pre3T1A (13) when combined with pre1–1 or pre2 mutations,
which lead to defective chymotrypsin-like activity, significantly enhanced pre1–1- or pre2-induced defects. In agreement with these results, we now found that the pre3-2 mutation considerably strengthened pre1–1-induced temperature sensitivity. These data indicate that, when lacking one of the other activities, the PGPH activity may become important. Therefore, the necessity for the PGPH activity may rest in a redundancy function. Alternatively, it cannot be excluded that conditions may exist which require a proteasome with all proteolytic sites being active.

Similarly to pre3–2, pre3–3, and pre3–5 mutations, the pre3–4 mutation did not cause any defect in protein degradation nor any other phenotype. The pre3–4 mutation in addition to a defect in the PGPH activity caused reduction of the trypsin-like activity. However, both activities are reduced to only minor extents, and this may be the reason for the fact that pre3–4 does not lead to defective protein degradation. The mutation (G171D) resides in a region that is in close vicinity to the Pre3 and Pre4 subunits of the neighbor b-ring. Even though pre3–4 more strongly influenced the trypsin-like activity, the mutation is not in close contact to the Pre3 b-ring neighbor subunit Pup1 (11), which harbors the active site responsible for trypsin-like activity (13). As pre3–4 mutants showed normal chymotrypsin-like activity and in addition exhibited no other phenotype typically found in cells defective in proteasome function, this mutation does not seem to impair general formation of the 20 S proteasome complex. We assume that pre3–4 either influences the Pup1 subunit by a long distance effect or disturbs the local arrangement of the Pre3 Pre4 Pup1 subunit cluster.

The pre3–1 mutation (G15D) locates to a region that is in close contact to the Pre3 active site cleft as well as the domain responsible for interaction of the Pre3 protein with its b-ring neighbor Pup1. Even though pre3–4 more strongly influenced the trypsin-like activity, the mutation is not close contact to the Pre3 b-ring neighbor subunit Pup1 (11), which harbors the active site responsible for trypsin-like activity (13). As pre3–4 mutants showed normal chymotrypsin-like activity and in addition exhibited no other phenotype typically found in cells defective in proteasome function, this mutation does not seem to impair general formation of the 20 S proteasome complex. We assume that pre3–4 either influences the Pup1 subunit by a long distance effect or disturbs the local arrangement of the Pre3 Pre4 Pup1 subunit cluster.

FIG. 5. Temperature sensitivity of pre3 single and pre3–2 pre1–1 double mutant strains. A, pre3–2 and pre3–6 mutants are temperature sensitive: Strain WCG4a (PRE3), YRG11 (pre3–1), YRG12 (pre3–2), YRG13 (pre3–3), YRG14 (pre3–4), YRG15 (pre3–5), and YRG16 (pre3–6) were streaked on YPD agar medium and incubated at 30 °C and 37 °C for 2 days. B, pre3–2 strengthens pre1–1-induced temperature sensitivity: Strains YRG12 (pre3–2), YHI29/1 (pre1–1), and YHI54/3211 (pre1–1, pre3–2), were streaked on YPD agar plates and incubated at 37 °C (top) or 30 °C (bottom) for 2 days.

FIG. 6. Temperature sensitivity of pre3–1 and pre3–6 cells is cell cycle-independent. DNA contents of WCG4a (PRE3), YRG11 (pre3–1), YRG12 (pre1–2), and YRG16 (pre3–6) cells as seen by fluorescence-activated cell sorting analysis after 0, 2, and 5 h of incubation at 37 °C. pre3–1 and pre3–6 cells show retarded transition through G2/M phase. All strains show heat shock-induced increase of 1n DNA content after a 2-h shift to 37 °C. WCG4a and YRG12 DNA contents are balanced to normal ratio after 5 h of incubation at 37 °C. In contrast, YRG11 and YRG16 cells remain at the 2-h 1n/2n DNA ratio, even at 5 h of incubation, but do not arrest in a specific phase of the cell cycle.

FIG. 7. pre3–1 and pre3–6 mutant strains form abnormally shaped cells. Misshapen cells found in YPD cultures (72 h/30 °C) of strain YRG16 (pre3–6) were photographed with a Zeiss Axioskop. The bar represents 20 μm.
alteration influencing the two active sites located within these subunits.

pre3–6 harbors a short deletion (Lys-85 to Glu-88) of the C-terminal part of the H2 helix. This region is directly connected to the H2-S5 loop, which forms the Pre3 part of the β-ring gate (the so-called β-annulus; Ref. 11) located at the central channel of the 20 S proteasome. pre3–6 causes significant reductions of all three peptide cleaving activities. We therefore speculate that pre3–6 may lead to a severe alteration of the complete 20 S proteasomal β-ring structure or may impair assembly of the 20 S complex.

Both mutations, pre3–1 and pre3–6, cause a deficiency in protein degradation as proven by stabilization of undefined ubiquitinated proteins as well as defined substrates of the proteasome pathway. In addition, these mutations resulted in pleiotropic phenotypes as temperature sensitivity and distinct cell cycle related effects. Although nearly identical, phenotypes seem to be slightly stronger expressed in pre3–6 mutants. We assume that both mutations lead to a general defect in proteasomal protein degradation. As indicated by the enhanced accumulation of ubiquitinated proteins at 37 °C, the temperature sensitivity of pre3–1 and pre3–6 cells can most probably be explained by insufficient removal of abnormal proteins generated under such heat stress conditions. In addition, in contrast to certain 19 S regulatory complex mutants (cim3/cim5) which, at restrictive temperature, stop growth in the G2/m phase (54), temperature sensitivity of pre3–1 and pre3–6 cells was proven to be a cell cycle-independent event.

At permissive temperature, pre3–1 and pre3–6 mutants contained increased amounts of 2n DNA indicating a disturbed time course of cell cycle phases. This result may be explained by stabilization of certain cell cycle regulators. Cells expressing truncated proteolytically stabilized versions of G1 cyclins Cln3 or Cln2 (55, 56) or cells that due to a grr1 mutation show defective Cln degradation (57) prematurely enter S phase with a reduced G1 phase. In addition, progress through and exit from mitosis essentially requires proteasomal destruction of B-type cyclins (58, 59) and other mitotic regulators as Pds1 (33) or Ase1 (35). Therefore, enhanced amounts of 2n DNA containing cells detected in pre3–1 and pre3–6 mutant strains may be assigned to premature entry into a new cell cycle as well as delayed transition through mitosis. However, start into a new cell cycle also requires destruction of the CDK inhibitor Sic1 via the proteasome. Therefore, premature entry into S-phase may not occur in pre3–1 and pre3–6 cells. This view is supported by the fact that no significant decrease in cell size was observed for pre3–1 and pre3–6 mutants as is normally found for budding yeast cells showing accelerated transition through G1.

Ectopic expression of G- and B-type cyclins in pre3–1 and pre3–6 mutant cells resulted in slightly to significantly reduced growth. In the case of B-type cyclin overexpression, the growth defect observed can most probably be attributed to impaired inactivation of B-type cyclin-dependent CDKs by this strengthening delayed exit from mitosis. Interestingly, stronger growth defects were found when overexpressing G1 cyclins in pre3–1 and pre3–6 mutant cells. However, no stringent necessity for G1 cyclin destruction could be detected so far (57). Therefore, sensitivity to ectopic expression found in pre3–1 and pre3–6 cells cannot be assigned to a defined process yet. Overexpression of Cln2 in wild type cells was found to result in hyperpolarization of cortical actin and securin indicating that Cln1/2-dependent CDKs trigger polar bud growth (60). In agreement with these results, impaired degradation of Cln1/2 in grr1 mutant cells resulted in the formation of elongated cells (57). Therefore, appearance of misshapen cells with strongly elongated buds in cultures of pre3–1 and pre3–6 mutants may be explained by delayed degradation of Cln1/2 proteins.
Mutations in the Yeast Proteasome β-Type Subunit Pre3 Uncover Position-dependent Effects on Proteasomal Peptidase Activity and in Vivo Function
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