PRE2, Highly Homologous to the Human Major Histocompatibility Complex-linked RING10 Gene, Codes for a Yeast Proteasome Subunit Necessary for Chymotryptic Activity and Degradation of Ubiquitinated Proteins*

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We have cloned the yeast PRE2 gene by complementation of pre2 mutants, which are defective in the chymotrypsin-like activity of the 20 S proteasome (multicatalytic-multifunctional proteinase complex). The PRE2 gene, a β-type member of the proteasomal gene family, is essential for life and codes for a 287-amino acid proteasomal subunit with a predicted molecular mass of 31.6 kDa. Missense mutations in two pre2 mutant alleles were identified. They led to enhanced sensitivity of yeast cells against stress. At the same time, pre2 mutants accumulated ubiquitinated proteins. The Pre2 protein shows striking homology to the human Ring10 protein (60% identity excluding the 70 amino-terminal residues), which is encoded in the major histocompatibility complex class II region. It represents a component of the low molecular mass polypeptide complex, previously shown to be a special type of the 20 S proteasome. The low molecular mass polypeptide complex is assumed to be involved in antigen presentation, generating peptides from cytosolic protein antigens, which are subsequently presented to cytotoxic T-lymphocytes on the cell surface. The high homology of Pre2 to Ring10 implies the hypothesis that Ring10 is a subunit of the low molecular mass polypeptide complex central in its chymotryptic activity. One might further suggest that replacement of constitutive proteasomal components by functionally related, stress-induced, and abnormal proteins. Among the proteins degraded are those of the ubiquitin-mediated proteolytic pathway (17, 18).

Experiments in yeast are gradually uncovering general functions of the enzyme complex in cellular life (17, 18). Here, the proteasome is found to function in degradation of short-lived, stress-induced, and abnormal proteins. Among the proteins degraded are those of the ubiquitin-mediated proteolytic pathway (17, 18).

These studies were initiated through analysis of mutants defective in proteolytic functions of proteasome yseE, the yeast proteasome. Three isolated mutants define two complementation groups, PRE1 and PRE2, and show a defect exclusively in the chymotrypsin-like activity of the enzyme particle (17, 19). The trypsin-like and the peptidylglutamyl peptide hydrolyzing activities remain unaffected. PRE1 was cloned and sequenced and was shown to encode an integral proteasomal subunit essential for cell viability (17, 19).

Here, we report on the essential yeast proteasomal gene PRE2. The PRE2 gene product shows striking structural similarities to the Ring10 protein, which imply related functions of both subunits within yeast and human proteasomes, i.e., a participation in their chymotryptic activity. As in pre1 mutants, the defect in the chymotrypsin-like activity of pre2 mutant proteasomes leads to hypersensitivity of cells to the amino acid analogue canavanine and to stress-induced accumulation of ubiquitinated proteins.

**EXPERIMENTAL PROCEDURES**

**Isolation of Mutants**—Mutants of proteasomal chymotrypsin-like activity were isolated on Petri dishes after ethyl methanesulfonate mutagenesis of strain c53-ABY5-86 (MATα pral-1 prbl-1 prcl-1).

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†‡ The abbreviations used are: MHC, major histocompatibility complex; Cbz, benzoyloxycarbonyl; kbp, kilobase pair(s).
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cpsl-3  ura3Δ5  leu2-3,112  his4Δ) using Cbz-Gly-Gly-Leu-4-nitroanilide as substrate (17). The 4-nitroaniline released by enzymatic hydrolysis was detected by a coupling reaction with N-(1-naphthyl)ethylenediamine yielding a pink color. Non-staining or weakly staining colonies were picked and genetically analyzed as described in Ref. 17. In addition, the PRE2 locus was complemented with plasmids carrying the human RING10 cDNA (Ref. 18). The resulting heterozygous diploids were sporulated, and asci were subjected to tetrad dissection. Of each tetrad, viability of only two spores harboring a wild-type type was tested. A phenotype was observed in one or two spores. 

For immunological detection of ubiquitin-protein conjugates, strains were grown in liquid YPD medium to early stationary phase at 30 °C and subsequently incubated at 37 °C for 3 h. After harvesting and washing, cells were resuspended in distilled water to yield a 50% (v/v) suspension, and washed glass beads, equivalent to the respective cell volume, were added. Cells were heated for an additional 10 min at 95 °C in Eppendorf tubes and subsequently vortex-mixed for 10 s with intermittent heating for 30 s. SDS-EDTA solution (4.5% SDS, 2.25 mM EDTA), equivalent to the cell volume, was added, and the samples were heated for an additional 10 min at 95 °C. Samples were centrifuged and the supernatants were used for immunoblotting. For SDS-polyacrylamide gel electrophoresis (10% gels), 50 μg of protein were applied onto each lane. Immunoblotting using ubiquitin-protein conjugate antibody was done as described in Ref. 17. Quantification of the dye produced by the peroxidase linked to the rabbit-immunoglobulin antibody was done using the JAVA 1.3 video analysis system (Jandel, Corte Madera, CA).

Gene Cloning and Analysis—Standard molecular biological (20) and yeast genetic/microbiological techniques (20-22) were used. The PRE2 gene was cloned by complementation of the defective chymotrypsin-like activity of a pre2-2 mutant strain using a yeast genomic library in the CEN4-ARS-URA3 shuttle vector YCP50 (23). One recombinant YCP50 plasmid restored the proteolytic activities of both the pre2-1 and the pre2-2 mutant. The complementing portion of its 12-kbp genomic insert was limited to 1.65 kbp after cloning of subfragments into the CEN1-ARS-URA3 shuttle vector pDP83.2 For sequence analysis of the PRE2 locus (see Fig. 1A) by the dideoxy chain termination method, pDP83-based plasmids with overlapping inserts from the PRE2 region served as template.

Construction of plasmids carrying a deletion of the PRE2 coding region started from a pDP83-based plasmid harboring a 0.9-kbp fragment from the 5′-flanking region of PRE2, which had been generated by exonuclease digestions (left bar in Fig. 1A). This fragment was inserted into the upstream of the pre2-1 and the pre2-2 alleles determined and a base-base exchange was detected in each allele (see Fig. 1B). Chromosomal deletion of the cloned pre2-1 and pre2-2 alleles was introduced after 5′ of the start codon was deleted, and a heterologous DNA was inserted at the site of the deleted DNA. The resulting deletion was verified by Southern blot analysis. The deletion of the PRE2 gene by one-step gene disruption (22) was done as follows. Plasmid pDP83ΔE2 was cut with BamHI, and a 1.7-kbp BamHI fragment carrying the HIS3 gene was inserted. The diploid strain SBY8/18 (MATa/MATα his3-11,15 his5-11,15 leu2-3,112/leu2-3,112 ura3Δ5/ ura3Δ5 can1/ can1) was transformed with the pre2Δ2 gene, and the resulting transformants were selected and retained. The ORF was amplified by PCR from the plasmid, and the resulting fragment was used to replace one chromosomal PRE2 copy with the deleted allele was verified by Southern blot analysis. The deletion of the PRE2 gene was observed in two independent transformants.

Construction of Isogenic pre Mutant Strains—Isogenic single-mu
tant strains were generated by introduction of the cloned pre1 or pre2 alleles into wild-type strains WCG44a or WCG44a (MATa or MATa his3-11,15 leu2-3,112 ura3Δ5) by the two-step gene replacement method (22). The resulting mutant strains were used for generation of isogenic double mutants by mating of a pre1-1 strain with a pre2-1 or pre2-2 strain, respectively. The peptidylglutamyl peptide-splitting activity of the proteasome was tested in the supernatants as described in Ref. 17 using Cbz-Gly-Gly-Leu-4-nitroanilide and Cbz-Leu-Leu-Glu-3′-naphthylamide, respectively, as substrates.

For immunological detection of ubiquitin-protein conjugates, strains were grown in liquid YPD medium to early stationary phase at 30 °C and subsequently incubated at 37 °C for 3 h. After harvesting and washing, cells were resuspended in distilled water to yield a 50% (v/v) suspension, and washed glass beads, equivalent to the respective cell volume, were added. Cells were heated for an additional 10 min at 95 °C. Samples were centrifuged and the supernatants were used for immunoblotting. For SDS-polyacrylamide gel electrophoresis (10% gels), 50 μg of protein were applied onto each lane. Immunoblotting using ubiquitin-protein conjugate antibody was done as described in Ref. 17. Quantification of the dye produced by the peroxidase linked to the rabbit-immunoglobulin antibody was done using the JAVA 1.3 video analysis system (Jandel, Corte Madera, CA).

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For dideoxy chain termination method, pDP83-based plasmids with overlapping inserts from the PRE2 region served as template.

RESULTS AND DISCUSSION

The PRE2 gene was cloned from a yeast genomic library (23) by complementation of the defective chymotrypsin-like activity in the two pre2 mutants isolated. Sequence analysis of the complementing DNA region revealed an open reading frame that encodes a 287-amino acid protein with a predicted relative molecular mass of 31.6 kDa (Fig. 1).

Data bank search found the nucleotide sequences situated upstream of the PRE2 gene to match almost perfectly those of the RPL16B gene from Saccharomyces carlsbergensis (24). RPL16A and RPL16B are duplicated genes encoding ribosomal proteins and are found in S. cerevisiae as well (25). The distance between the translation initiation codons of the divergently transcribed PRE2 and RPL16B genes amounts to 600 nucleotides (Fig. 1). Whether these two genes share common regulatory sites is unknown.

Standard genetic procedures (22) revealed the two pre2 mutant genes to be alleles of the isolated PRE2 gene. The cloned PRE2 DNA was able to direct integration of a selectable marker gene to the wild-type locus corresponding to the pre2 mutant alleles (not shown). Furthermore, the pre2-1 and pre2-2 mutant alleles were cloned (see “Experimental Procedures”) and shown to confer a defective proteasomal chymotryptic activity to wild-type cells. Both mutant genes contain a missense mutation each (Fig. 1B).

PRE2 is essential for cell proliferation. Sporulation of heterozygous diploids carrying a null allele of PRE2 (Fig. 1A) produces asc of which only the two spores harboring a wild-type PRE2 gene grow up to visible colonies. Although the pre2 null mutant spores are capable of germinating, they arrest after two to three cycles of cell division. An identical cell division arrest is seen with spores deleted in the PRE1 gene (not shown). Seven of eight other hitherto cloned yeast proteasomal genes (26-30) are essential for life as well. Our results with PRE2 confirm the importance of a complete proteasomal particle for cell survival.

Biochemical analysis of pre mutants with respect to their defect in the chymotrypsin-like activity revealed a reduction in pre2-2 mutants of 95%, which is similar to the value found in pre1-1 mutants (Table I; Ref. 17). Pre2-1 mutants exhibit a 3-fold higher residual activity compared with pre2-2 and pre2-1 mutants (Table I). Introduction of the pre2-1 mutation into a pre1-1 mutant strain leads to a slight additional decrease in the chymotrypsin-like activity compared with pre1-1 single mutants. Combination of the pre2-2 mutation with the pre1-1 mutation in a double mutant finally yields a residual activity of only 4% (Table I).

As shown previously (17), the peptidylglutamyl peptide-splitting activity remains in the pre2 and pre2 mutants. Interestingly, this activity is even increased in these mutants, most strongly in those carrying the pre1-1 allele (Table I).

D. Pridmore, unpublished data.
**TABLE 1**

| Strains investigated are isogenic except for the respective pre mutation(s) as indicated. For strain construction, preparation of cell extracts, and enzyme tests see "Experimental Procedures." Values presented are means of at least three independent determinations.

| Mutation | Activity against Cbz-Gly-Gly-Leu-4-nitroanilide | Activity against Cbz-Leu-Leu-Glu-β-naphthylamide |
|----------|-----------------------------------------------|--------------------------------------------------|
| Wild type | 100                                           | 100                                              |
| prel-1   | 5.5 ± 1.5                                     | 153 ± 17                                         |
| prel-2   | 15.2 ± 2.3                                    | 117 ± 14                                         |
| pre2-1   | 5.0 ± 1.1                                     | 119 ± 14                                         |
| pre2-2   | 4.9 ± 0.9                                     | 149 ± 36                                         |
| pre2-2/prel-1 | 4.0 ± 0.6                                | 145 ± 23                                         |

**Fig. 2.** Effects of elevated temperature and canavanine application on growth of pre mutant cells. Cells from isogenic strains differing in the pre mutations as indicated (see "Experimental Procedures"; WT, wild type) were streaked for single colonies onto YPD agar plates (a, b) or onto MV-agar plates (0.68% yeast nitrogen base without amino acids, 2% glucose, 2% agar, and supplements as required (21)) containing no (c) or 0.4 mg/ml (d) canavanine sulfate. Plates were incubated at 30 °C (a, c, d) or 38 °C (b) for up to 2 days.

Because of the high activity of other proteases against the substrate of the trypsin-like proteasomal activity (Cbz-Ala-Arg-4-methoxy-β-naphthylamide) in crude extracts, effects of the prel and pre2 mutants on this activity could not be determined.

Cells defective in chymotrypsin-like activity were reported to have exhibited sensitivity to stresses, as were elevated sensitivity to stresses, as were elevated temperature of 30 °C on YPD and, more pronounced, on MV medium. MV media is affected by the "prel-1" mutation (Fig. 2, a and c).
The stress conditions mentioned were shown to lead to accumulation of ubiquitinated proteins in pre1-1 mutant cells (17). In part, similar phenotypes are induced by mutations residing in the PRE2 gene. With its high residual chymotrypsin-like activity, a pre2-1 mutant strain does not exhibit heat and canavanine sensitivity (Fig. 2, b and d). However, when the pre2-1 mutation is combined with the pre1-1 mutation, the resulting double mutants show phenotypes that are by far more dramatic than those observed for the pre1-1 single mutant (Fig. 2, a-d). In contrast to pre2-1 single mutants, pre2-2 mutants exhibit some canavanine sensitivity (Fig. 2d), but as pre2-1 mutants, they are not heat-sensitive (Fig. 2b).

Combination of pre2-2 with pre1-1 yields double mutants, which also show strengthening of the pre1-1-induced phenotypes (Fig. 2, a-d). Remarkably, pre2-1, which causes a less pronounced phenotype compared with pre2-2, induces a somewhat tighter phenotype when combined with pre1-1 as does pre2-2.

Analysis of the accumulation of ubiquitinated proteins in the mutant strains parallels their stress-dependent growth behavior. While no accumulation is seen in pre2-1 mutant cells upon stress application (not shown), pre2-2, pre1-1, and pre1-1/pre2-2 mutants exhibit a 3-, 9-, and 18-fold increase, respectively, in the amount of high molecular weight ubiquitin-protein conjugates compared with wild type (Fig. 3). We consider this accumulation of ubiquitinated proteins in the pre mutant cells to result from reduced degradation. This view is supported by the finding that hydrolysis of short-lived N-end rule substrates, which are known to be destined for degradation by attachment of ubiquitin chains, is blocked in these mutants (18).

An additional phenotype of the pre mutants rests in their capacity to better survive a heat shock of 52°C when preincubated at 37°C (not shown).

The Pre2 protein is shown to be an integral component of the yeast proteasome by two lines of evidence. 1) The reduction of chymotrypsin-like activity is a feature inherent to purified pre2 mutant proteasomes (17). 2) The Pre2 protein shows structural similarity to proteins from yeast (including Pre1, see Fig. 4) and other organisms, which have been unambiguously identified as components of the proteasome particle. All identified proteasomal genes encode subunits with characteristic similarities and can be classified as a proteasomal gene family of ancient origin (5, 31). PRE2 clearly represents a member of this family.

An extraordinarily high degree of homology is found between the Pre2 protein and the recently analyzed RING10 gene product, which is encoded in the human MHC class II region. Not considering the poorly conserved amino termini, a striking 60% identity and 80% similarity can be established in the entire remaining three-quarters of the PRE2- and RING10-encoded proteins (Fig. 4). Because identity between proteasomal proteins of a given species generally does not exceed values of 40%, stronger homologies between two proteasome subunits of different origin indicate corresponding functions of the respective subunits within the heterologous protease complexes. Thus, the highly conserved structures of the Pre2 and Ring10 proteins from evolutionarily distant organisms, i.e. yeast and man, predict some functional equivalence of these two subunits.

The alignment of the Pre2 and Ring10 protein sequences in Fig. 4 includes other yeast (Pre1 and Pup1 (17, 28)) and human (Ring12 and β (15, 32)) proteasome subunits as well as the β-subunit of the proteasome from the archaeabacterium Thermoplasma acidophilum (31). These seven proteins can be grouped into the β-subfamily of proteasome components, of which the archaeabacterial β-subunit is regarded as the ancestor (31). The only two types of subunits, α and β, constituting the Thermoplasma proteasome, solely confer chymotrypsin-like activity to the complex. This activity is thought to reside in the β-subunit (31). Remarkably, the amino acid exchanges in the pre2-1 (Gly-259 to Ser-259) and the pre2-2 (Ala-124 to Val-124) gene products both reside at positions highly conserved among the archaeabacterial and eukaryotic proteasomal proteins compared in Fig. 4.

None of the yeast proteasomal subunits involved in chymotrypsin-like activity, Pre1 and Pre2, exhibit any sequence similarity to known proteinases. By assuming some functional homology of Pre2 and Ring10, speculations about a new type of serine proteinase represented by Ring10 (14) cannot be supported by the Pre2 sequence data. No histidine exists in the Pre2 region corresponding to the Ring10 sequence stretch that was found to contain a histidine residue in a similar environment as the one present in the catalytic triad of subtilisin-type proteinases. Two recently cloned genes from the rat and the mouse (33, 34), which encode proteasomal subunits (RC1 and MC13, respectively) highly homologous to Ring10, also lack a histidine at this position. The chymotrypsin-like activity of the proteasome could be brought about by a completely new proteinase type, which might even be formed by interaction of two or more subunits. The fact that mutations in two different subunits of the yeast proteasome result in defective chymotrypsin-like activity may point to a cooperation of distinct proteasomal proteins in active-site formation.

Mutations in yeast proteasomal subunits have uncovered proteasome functions in a variety of cellular events such as growth, differentiation, and stress response (this paper (17)). One molecular basis of action seems to reside in the capacity to degrade ubiquitinated proteins (this paper (17, 18)), which are derived from different degradation pathways (18). In mammalian cells, the proteasome is also expected to exert a variety of different functions, a specialization of which is the hypothetical participation in antigen processing. The striking homology between the human MHC-encoded Ring10 protein and the proteasomal Pre2 subunit, necessary for the chymotrypsin-like activity of the yeast protease complex, supports the involvement of human proteasomes in this latter process.

Whether ubiquitin conjugation to proteins is a prerequisite for cleavage and subsequent presentation of peptides as antigens has to be investigated.

In contrast to Pre2, the Ring10 subunit does not seem to...
be a constitutive proteasomal protein, because the complete absence of this MHC-encoded subunit from human cells does not impair its viability (11). Therefore, it may be assumed that MHC-encoded proteasomal subunits substitute for constitutive components of similar structure. These constitutive components may provide the proteasome with similar catalytic specificity but they change the intracellular biological function of the particle. Incorporation of Ring10 could generate a proteasome that serves the special task of antigen processing, whereas an equivalent constitutive subunit may provide the proteasome with similar catalytic activity.

**Fig. 4.** Alignment of the Pre2 protein sequence with other \( \beta \)-type proteasomal proteins from the archaeabacterium \( T. acidophilum \) (Taș, Ref. 31), the yeast \( Saccharomyces cerevisiae \) (ScPre1 and ScPup[17,28]), and man (HsRing10, HsRing12, and Hs2) (14, 15, 32). Note that the Hs sequence is assembled with residues encoded by a cDNA lacking the 5'-end of the coding region and an overlapping 3'-end sequence derived from amino acid sequencing. Some of the amino-terminal peptides (NT) obtained from amino acid sequencing of proteasomal subunits from man (Hs [35]) and rat (Ro, Rn1, and Rn2 [36]) are included (x, unidentified residues). Sequences are shown in single-letter code; gaps (−) are inserted for optimal alignment. Amino acids are depicted in boldface type if more than half of the residues are identical. Residues identical or conserved at corresponding positions in ScPre and HsRing10 are marked with SCPUPl 11: 8cPr.l

| Taș | 1: | mqlTptdttesvteslVesAp1dtttltptpsacgfgLeptefffaglggverkq-1-4HdGTTLRbKfWbOVbDAbLbWcAbewLvS | 1: | mqlTptdttesvteslVesAp1dtttltptpsacgfgLeptefffaglggverkq-1-4HdGTTLRbKfWbOVbDAbLbWcAbewLvS |
| ScPre1 | 1: | mqlTptdttesvteslVesAp1dtttltptpsacgfgLeptefffaglggverkq-1-4HdGTTLRbKfWbOVbDAbLbWcAbewLvS | 1: | mqlTptdttesvteslVesAp1dtttltptpsacgfgLeptefffaglggverkq-1-4HdGTTLRbKfWbOVbDAbLbWcAbewLvS |
| ScPup[17,28] | 1: | mqlTptdttesvteslVesAp1dtttltptpsacgfgLeptefffaglggverkq-1-4HdGTTLRbKfWbOVbDAbLbWcAbewLvS | 1: | mqlTptdttesvteslVesAp1dtttltptpsacgfgLeptefffaglggverkq-1-4HdGTTLRbKfWbOVbDAbLbWcAbewLvS |

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