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PRE3, highly homologous to the human major histocompatibility complex-linked *LMP2* (*RING12*) gene, codes for a yeast proteasome subunit necessary for the peptidylglutamyl-peptide hydrolyzing activity

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Abstract

20S proteasomes are multifunctional proteinase complexes ubiquitous in eucaryotes. We have cloned the yeast *PRE3* gene by complementation of the *pre3–2* mutation, which leads to a defect in the peptidylglutamyl-peptide hydrolyzing activity of the 20S proteasome. The *PRE3* gene, a β-type member of the proteasomal gene family, is essential for cellular life and codes for a 193-amino acid proteasomal subunit with a predicted molecular mass of 21.2 kDa. The Pre3 protein shows striking homology to the human proteasome subunits Hsδ and Lmp2 (Ring12). Lmp2 is encoded in the major histocompatibility complex class II region implicating proteasomes in antigen processing.

Key words: Proteasome; Proteolysis (yeast); Antigen processing (human MHC)

1. Introduction

20S proteasomes are abundant proteinase complexes (molecular mass about 700 kDa) in the cytoplasm and nucleus of eucaryotic cells, highly conserved from yeast to man [1,2]. They are composed of a variable set of non-identical but structurally related subunits (molecular masses ranging from 20 to 35 kDa) arranged in a hollow cylindrical structure. 20S proteasomes exhibit multiple, distinct catalytic activities including peptide bond cleavage on the carboxyl side of hydrophobic, basic and acidic amino acid residues. Three types of proteolytic activity have been referred to as chymotrypsin-like, trypsin-like and peptidylglutamyl-peptide hydrolyzing (PGPH) activities, respectively [3,4,5].

20S proteasomes act as the proteolytic core of a higher molecular mass complex (about 1,5 MDa), the 26S proteasome. In vitro studies demonstrated that modification by ubiquitin targets proteins for energy dependent degradation via the 26S complex [6,7].

In mammals, the presentation of intracellular proteins to the immune system requires their degradation to small peptides that then become associated with major histocompatibility complex (MHC) class I molecules [8,9]. The antigenic peptides for MHC class I assembly may be provided by a ubiquitin-mediated proteolytic pathway [10]. Two proteasomal subunits, the human proteins Ring10 [11] and Ring12 [12] and their murine equivalents Lmp7 [13] and Lmp2 [14], respectively, are encoded within the MHC class II region, implicating special functions of proteasomes in antigen processing. In cells lacking these MHC-encoded proteasome subunits the efficiency of antigen processing should be reduced [15,16,17].

Yeast mutants *pre1–1* and *pre2–2* deficient in the chymotrypsin-like activity and *pre4–1* defective in the peptidyl glutamyl peptide hydrolyzing activity of the proteasome have given evidence that proteasomes are involved in the ubiquitin-mediated proteolytic pathway in vivo [18–22]. Proteasomes were found to function in the degradation of the short-lived N-end rule protein substrates [19,20], metabolically regulated enzymes (Schork, S., Bee, G., Thumm, M. and Wolf, D.H., submitted) and abnormal proteins [23,24]. Proteasomes also have been shown to be involved in cell cycle control [25–27]. Three yeast genes, *PRE1*, *PRE2*, and *PRE4*, encoding proteasomal subunits necessary for proteolytic activities of the proteasome were cloned and sequenced. Each of them is essential for cell viability [18,21,22].

Here, we report on the cloning of the essential yeast *PRE3* gene by complementation of the *pre3–2* mutation, which leads to a defect in the peptidylglutamyl-peptide hydrolyzing (PGPH) activity of the proteasome. The *PRE3* gene product shows striking homology to the human proteasome subunits Hsδ [28] and Lmp2

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Abbreviations: Cbz, benzyloxycarbonyl; βNA, β-naphthylamine; MHC, major histocompatibility complex; PGPH, peptidylglutamyl-peptide hydrolyzing.

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(Ring12) [12] which is located in the MHC class II region.

2. Materials and methods

2.1. Yeast strains and manipulation

Yeast *S. cerevisiae* strains were grown in complete (YPD) or minimal (SD) media prepared according to [29]. For genetic and molecular biological techniques standard protocols were used [30–32]. For transformation a protocol modified by Heinemeyer et al. [18] was followed.

Yeast strains FABYSD-17C (*MATa kex2 pra1–1 prb1–1 prc1–1 cps1–3 prd1⁻ his⁻ lys⁻ ade⁻*) [21] and WCG4 (*MATa/MATα* his3–11,15/his3–11,15 leu2–3,112/leu2–3,112 ura3/ura3 can^S/can^S) [22] were used as proteinase yscE wild type. The mutant strain C13–9C (*MATα* pre3–2 prb1–1 prc1–1 ura3Δ5 his⁻ lys⁻) is a derivative from the *pre3–2* mutant strain C13 [33].

2.2. Isolation of mutants

Mutants of the proteasomal PGPH activity were isolated after mutagenesis with ethylmethane sulfonate of strain FABYSD-17C using an in situ test with Cbz-Leu-Leu-Glu-βNA as substrate [21,34]. The β-naphthylamine released from the substrate by the PGPH activity was detected by coupling it to Fast Garnet GBC yielding a red azo dye. Pale mutant colonies were picked. Four mutant strains, C6 (*pre3–1*), C13 (*pre3–2*), C19 (*pre3–3*) and C35 (*pre4–1*), were isolated and biochemically and genetically analyzed [21,34].

2.3. Gene cloning and analysis

The *PRE3* gene was cloned by complementation of the defective PGPH activity of the *pre3–2* mutant strain C13–9C using a yeast genomic library in the *CEN4-ARS1-URA3* shuttle vector YCp50 [35]. One recombinant YCp50 plasmid restored the PGPH activity in the *pre3–2* mutant strain. Restriction mapping revealed a 6.9 kbp genomic DNA insert. The complementing DNA sequence was limited to 3.2 kbp after cloning of subfragments into the vector YCp50 (Fig. 1). For sequence analysis the complementing DNA was cloned as an *Eco*RV fragment in both directions into the *Eco*RV-cut vector pBluescript SK (Stratagene). Stepwise deletions nesting the insert of each pBluescript SK-based plasmid were generated by the Exonuclease III/Exonuclease VII digestion method [36]. The plasmid was cut at the unique polylinker sites *Bam*HI and *Sac*I and then digested with Exonuclease III/Exonuclease VII (Boehringer Mannheim, Germany), yielding a series of pBluescript SK-based plasmids with DNA segments overlapping the *PRE3* region. These plasmids served as template DNA. Nucleotide sequencing of both DNA strands was performed using the T7-Polymerase DNA-Sequencing Kit from Pharmacia (Freiburg, Germany) and the reversed primer which binds adjacent to the polylinker.

2.4. Chromosomal deletion of PRE3

A 2.3 kbp *Eco*RI–*Sal*I fragment harboring the *PRE3* gene was cloned in the vector pSP64 yielding pJK2. Plasmid pJK2 was digested at the polylinker sites *Hin*dIII and *Sal*I, the ends were filled up using the Klenow reaction and religated to yield pJK3. Plasmid pJK3 was linearized using the PstI site located in the *PRE3* gene and digested with exonuclease Bal31 until 248 bp were removed between the ends. The ends were religated using a *Bam*HI linker yielding pJK6. The *PRE3* deletion (Figs. 1 and 2, nucleotides from position 131 to 379) was verified by sequencing. A 1.8 kbp *Bam*HI fragment containing the *HIS3* gene was inserted into the *Bam*HI-cut pJK6 yielding the *PRE3* deletion allele (Fig. 1). The *pre3Δ*::HIS3 deletion allele was transformed as a 3 kbp *Eco*RI–*Mlu*I fragment into the histidine-auxotrophic diploid wild type strain WCG4. His⁺ transformants were selected. The replacement of one of two copies of *PRE3* with the dabeletion allele was confirmed by Southern blot analysis (one step gene disruption; see [32]). The *PRE3/pre3Δ*::HIS3 heterozygous diploids were sporulated, and asci were subjected to tetrad analysis. Of each tetrad, viability of only two His⁻ spores was observed.

3. Results and discussion

We had isolated four mutants (C6, C13, C19 and C35) deficient in the peptidylglutamyl-peptide hydrolyzing (PGPH) activity of proteinase yscE, the yeast proteasome, via their defect in the activity against the peptide substrate Cbz-Leu-Leu-Glu-βNA [34]. The genetic analysis of these mutants revealed two complementation groups, *PRE3* and *PRE4*. The mutant alleles were called *pre3–1* (C6), *pre3–2* (13), *pre3–3* (C19) and *pre4–1* (C35) [21]. The mutants C13 (*pre3–2*) and C35 (*pre4–1*) were completely devoid of proteolytic activity against Cbz-Leu-Leu-Glu-βNA. The mutant strains C6 and C19 exhibited residual activities against Cbz-Leu-Leu-Glu-βNA of 17% and 30% as compared to wild type.

The *PRE3* gene was cloned from a yeast genomic library [35] by complementation of the peptidylglutamyl-peptide hydrolyzing (PGPH) activity of the *pre3–2* mutant strain C13–9C.

Sequence analysis of the complementing DNA region (Fig. 1) revealed an open reading frame that encodes a 193-amino acid protein with a predicted molecular mass of 21.2 kDa (Fig. 2).

Data bank search revealed, that the *PRE3* gene is located 370 bp downstream of the *CEN10* nucleotide sequence [37] as shown in Fig. 1. Therefore, *PRE3* is mapped to the right arm of chromosome X.

The chromosomal deletion of the *PRE3* gene is lethal. Sporulation of heterozygous diploids (*PRE3/pre3Δ*::HIS3) yielded two wild type spores which grew up to colonies and two null mutant spores which germinated, but were unable to proliferate. Thus the *PRE3* gene product like the hitherto cloned proteasomal subunits

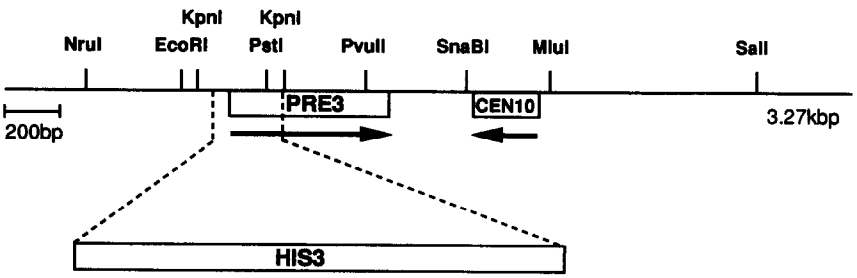

Fig. 1. Restriction map of the *PRE3* locus and deletion. The 1.4 kbp *Nru*I–*Sna*BI fragment represents the shortest DNA streach complementing the *pre3–2* mutation. Dashed lines indicate the region of *PRE3*, that was replaced with *HIS3* (see section 2).

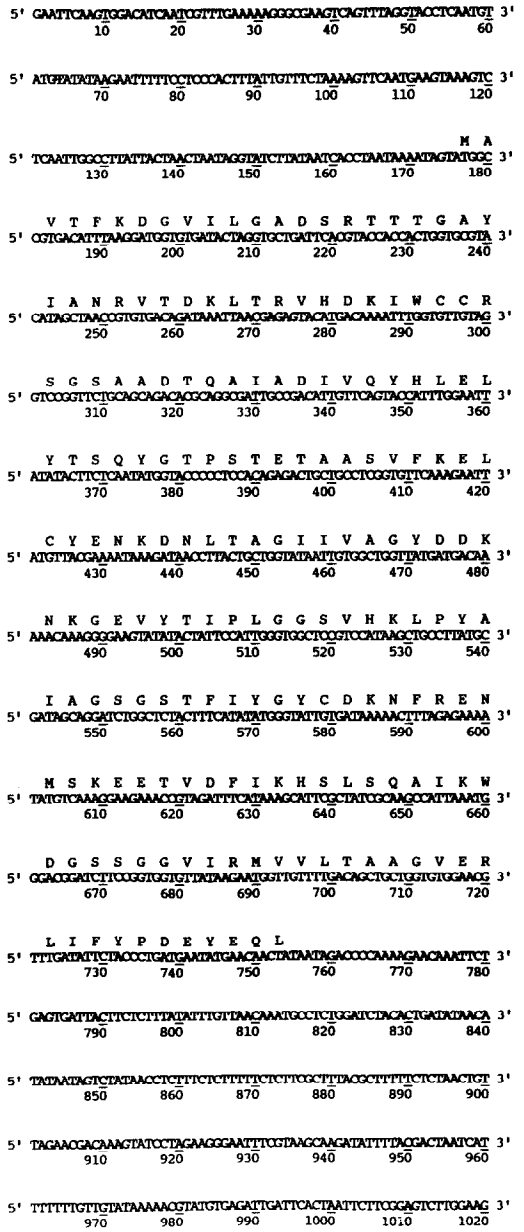

Fig. 2. Nucleotide sequence of the *PRE3* gene and deduced amino acid sequence of the Pre3 Protein.

necessary for proteolytic activities of the proteasome [18,21,22] was proven to be essential for cellular life.

Molecular analysis of the Pre3 protein and proteins of yeast and higher eucaryotes identified as components of proteasomes demonstrate that the *PRE3* gene product is a subunit of the yeast proteasome. The Pre3 protein can be classified as a member of the β-type proteasome gene family [38]. Thus the up to now identified subunits which can be linked to proteolytic activity of the complex are all of the β-type [18,21,22]. This gives evidence for the idea that the β-subunits of the 20S proteasome may exert catalytic functions whereas the α-subunits may be needed for the assembly and regulation of the 20S and/or 26S proteasome complex. Using the EMBL PROSITE database a sequence motif WDGSS which is typical for glutamine synthetases [39] was found within the Pre3 sequence located at the highly conserved βII region (Fig. 3). As glutamine synthetase also the Pre3 subunit may recognize or bind glutamyl residues as a part of the proteasomal PGPH-substrate. Besides the conserved domains characteristic for β-type proteasomal subunits no striking homology between the Pre3 protein and the gene products of *PRE1*, *PRE2* and *PRE4* was found (not shown).

The alignment of the Pre3 protein with the Hsδ subunit of the human proteasome (55% identity, 77% similarity) and the human MHC-encoded protein Lmp2 (Ring12) (44% identity, 69% similarity) is shown in Fig. 3. In human cells deletion of *LMP2* does not lead to lethality, whereas the *PRE3* gene is essential for the yeast cell. This result suggests that as Pre3 in yeast the most homologous subunit HSδ in human cells may perform house-keeping functions whereas the Lmp2 subunit may be needed for specific tasks, as is antigen processing. Recently it has been reported that deletion as well as γ-interferon induced expression of the MHCII-encoded Lmp2 and Lmp7 proteins changes the proteolytic activities of the respective proteasomes [15,16]. Pre3 as the

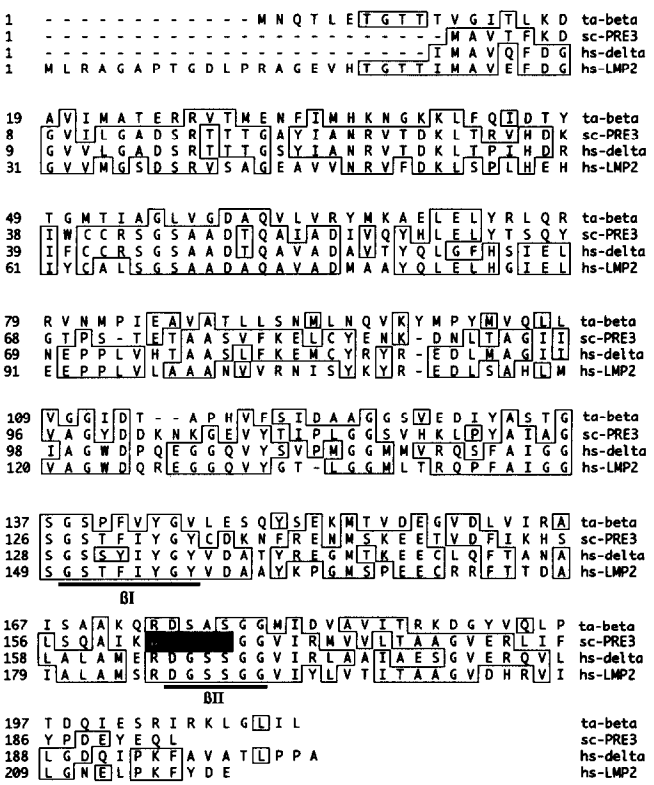

Fig. 3. Alignment of the β-subunit from archaebacteriae *T. acidophilum* [38], the yeast Pre3 protein, the Hsδ subunit of the human proteasome [28], and the human MHC-encoded Lmp2 (Ring12) protein [12]. Gaps are inserted for optimal alignment. Identical amino acids are indicated by boxes. The glutamine synthetase motif is shown by the filled box. The consensus PROS box domains defining members of the β-type proteasome family are underlined.

yeast homologue of Lmp2 is necessary for the PGPH-activity of the proteasome. This finding supports the idea that Lmp2 is needed for the catalytic function of the proteasome and modulates the proteolytic acivity to improve antigen processing.

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