Enzyme Protein 1993;47:189–201

Wolfgang Hilt
Wolfgang Heinemeyer
Dieter H. Wolf

Institut für Biochemie, Universität Stuttgart, Deutschland

Studies on the Yeast Proteasome Uncover Its Basic Structural Features and Multiple in vivo Functions

Key Words
Yeast
Proteasome
Proteolysis
Ubiquitin
Stress
Cell cycle
Cell regulation

Abstract
Proteasomes are large multicatalytic protease complexes found in the cytoplasm and nucleus of all eukaryotic cells. 20S proteasomes are cylindrically shaped particles composed of a set of different subunits arranged in a stack of 4 rings with 7-fold symmetry. In yeast 14 different genes are known, which are proposed to code for the complete set of 20S proteasomal subunits. They can be divided in 7α- and 7β-type subunits. 26S proteasomes are even larger proteinase complexes which contain the 20S proteasome as the functional proteolytic core. They degrade ubiquitinylated proteins in vitro. Several yeast 26S proteasome subunits have been characterized as members of a novel ATPase family. Studies with yeast 20S and 26S proteasome mutants uncovered the function of proteasomes in stress-dependent and ubiquitin-mediated proteolytic pathways. Proteasomes are important for cellular regulation, cell differentiation, adaptation to environmental changes and are involved in cell cycle control.

Activities, Genes and Proteins of the 20S Proteasome

In 1984 Achstetter et al. [1] purified a high molecular mass multisubunit proteinase from *Saccharomyces cerevisiae,* which was named proteinase yscE. Four years later the identity of this yeast enzyme with the multicatalytic proteinase complex or proteasome of other eukaryotes was established by an extensive comparison of its biochemical, structural and immunological features with those of the 20S cylinder particles from *Xenopus laevis* [2]. Proteinase yscE exhibits the typical proteaso-

Wolfgang Hilt
Institut für Biochemie
Universität Stuttgart
Pfaffenwaldring 55
D-70759 Stuttgart (Germany)

1019–6773/93/
0476–0189$8.00/0

Table 1. Genes of the yeast 20S proteasome and properties of their encoded proteins

Gene (other names)	Type	Chromosomal location	Predicted protein[a]		Motifs	Correlation with proteolytic activity
			molecular mass	pI		
PRS1 (YC-1)	α	XV	31.6	5.20	–	–
PRS2 (SCL1,YC-7α,Y8)	α	VII	28.0	6.05	NLS1	–
Y7	α	n.d.	27.2	5.54	2 × cAMP-PS	–
Y13	α	n.d.	28.7	4.96	2 × cAMP-PS, Tyr-PS	–
PUP2	α	n.d.	28.6	4.58	cAMP-PS	–
PRE5	α	XIII	25.6	7.30	Tyr-PS	–
PRE6	α	XV	28.4	7.27	cAMP-PS, Tyr-PS, NLS1	–
PRE1	β	V	22.5	6.01	NLS2	chymotryptic
PRE2 (PRG1)	β	XVI	31.6 (23.3)	6.02 (6.19)	Tyr-PS	chymotryptic
PRE3	β	X	21.2	5.37	Gln-Synthetase	PGPH
PRE4	β	VI	29.4 (25.1)	5.75 (5.84)	Tyr-PS, NLS1	PGPH
PRS3	β	II	27.1 (25.1)	6.11 (6.86)	cAMP-PS	–
PUP1	β	XV	28.3 (25.2)	6.60 (6.53)	–	-
PUP3	β	V	22.6	4.93	–	tryptic?

Except for NLS1, the motifs listed were found via search in the 'PROSITE' database. cAMP-PS = cAMP-dependent phosphorylation site; Tyr-PS = tyrosine phosphorylation site; NLS1 = nuclear localization signals of the SV 40 large T-antigen type; NLS2 = bipartite nuclear localization site; Gln-Synthetase = glutamine synthetase consensus sequence; PGPH = peptidylglutamyl peptide hydrolyzing.

[a] The molecular mass and pI values were calculated for the longest predictable translation products of the respective open reading frames. Values in brackets correspond to putative processed forms predicted from sequences of N-terminal peptides of homologous proteins from other species [20, 21].

mal cleavage activities towards chromo- and fluorogenic peptide substrates, i.e. it has a chymotrypsin-like, a trypsin-like and a peptidylglutamyl peptide-hydrolyzing (PGPH) activity [3]. Of the two additional activities described by Orlowski et al. [4] for the proteasome of bovine pituitary, only the branched chain amino acid-preferring activity is detectable in partially purified yeast proteasomes [Zimmermann and Hilt, unpublished results].

The subunit composition of proteinase yscE is as complex as in higher eukaryotes, showing a ladder of protein bands in the molecular mass range between 20 and 35 kD after SDS-PAGE, which can be separated into 14 protein spots after two-dimensional gel electrophoresis [3].

In the meantime the primary structures of 14 yeast proteasomal subunits have become known through cloning and sequencing of the corresponding genes (table 1). As found for all 20S proteasomal proteins from higher eukaryotes, all yeast subunits show structural relationships to each other and to those of other species. They can be grouped into 7 α-type and 7 β-type proteins according to their degree of homology to either the α or the β subunit of the *Thermoplasma* proteasome (table 1). The β-type genes *PRE1, PRE2, PRE3* and *PRE4* were cloned by complementation of mutants defective in the chymotryptic (*pre1* and *pre2* mutants) or the PGPH (*pre3* and *pre4* mutants) activity of the proteasome [3, 5–7]. *PRE2* was independently identified as a single copy suppressor (named *PRG1)* of the *crc1* mutation which causes a high frequency of chromosome loss [8]. Two other β-type genes were cloned by chance: *PUP1* [9], and an originally unidentified proteasomal

gene here referred to as *PUP3* [10], which has now turned out to be related to a gene encoding the C10II subunit of the rat proteasome [11]. The 7th β-type gene, homologous to the mammalian and *Drosophila* C5 subunit, is *PRS3* [12]. The 7 yeast proteasomal genes of the α-type are: *PRS1* [13] (formerly YC-1) and *PRS2* [13] (formerly YC-7α, independently cloned as Y8 [14] and as *scl1*$^+$, a dominant suppressor of the *crl3* mutation which confers cycloheximide resistance combined with temperature sensitivity [15]), Y7 and Y13 [14], *PUP2* [16], (also cloned accidently), and the recently identified genes *PRE5* and *PRE6* [17]. These 7 α-type genes encode a subfamily of proteasomal subunits with strongly conserved primary structures (up to 35% identity), whereas the β-type subunits represent a more divergent subfamily (up to 25% identity). With the exception of the gene encoding the Y13 subunit, the chromosomal deletion of each of the yeast proteasomal genes is lethal for the cell.

The chromosomal location of most of the 14 proteasomal genes was determined showing a random distribution over the yeast genome (table 1]. Nothing is known about regulatory elements allowing a coordinate expression of the genes.

The calculated molecular masses of the 14 yeast proteasomal subunits range from 21.2 to 31.6 kD, which is in agreement with the molecular masses of the protein bands appearing after SDS-PAGE of purified yeast 20S proteasomes [2, 18, 19]. A processing of yeast β-type subunits by cleaving at their N-termini, as shown for some β-type subunits of higher eukaryotes [20–23] as well as for the archaebacterial β-subunit [24], has not been documented so far.

None of the yeast proteasomal proteins exhibits homologies to any known type of protease. However, genetic studies connected certain β-type subunits with proteolytic activities of the yeast proteasome. Intact Pre1 and Pre2 proteins are necessary for the chymotrypsin-like activity [3, 5], intact Pre3 and Pre4 proteins are necessary for the PGPH activity [6, 7]. These results support the idea that proteolytically active sites may be formed by interaction of at least two neighboring subunits. On the other hand, it cannot be excluded that individual subunits within the proteasome might represent totally new types of proteolytic enzymes. If the latter were true, the loss of individual activities caused by mutations in either of two subunits would imply conformational forces acting in an inhibitory fashion from a mutated subunit on the activity of another catalytic subunit. A candidate for being involved in the trypsin-like proteasomal activity is Pup3, since its bovine homologue, subunit theta, was shown to be accessible to the trypsin protease inhibitor leupeptin [25]. Remarkably, all subunits which can be correlated with proteolytic activities are so far of the β-type. Thus, in analogy to the archaebacterial 'urproteasome' with its β-subunits forming the inner two rings of the cylindrical particle [26], the sites of proteolysis in eukaryotic proteasomes are most probably located in the central rings made up of β-type subunits.

In a general search for motifs in the primary structures of the yeast proteasomal subunits, several potential phosphorylation sites for tyrosine kinases and cAMP-dependent threonine/serine kinases were found, predominantly in the α-type subunits. Nuclear localization signals, which may play a role in the transport of whole proteasomes or single subunits to the nucleus, are contained in the Prs2, Pre6, Pre1 and Pre4 proteins [3, 7, 13, 17]. Interestingly, the Pre3 subunit as a candidate for carrying out the hydrolysis of peptidylglutamyl peptide bonds has a glutamine synthetase motif, which might be involved in recognition of glutamic acid residues [6] (table 1).

Evidence for a Set of 14 Different Subunits in Eukaryotic 20S Proteasomes

The now known 14 yeast proteasomal subunits, 7 each of the α- and the β-type, are likely to represent the complete set of proteins constituting the 20S core complex in this organism. Firstly, the 14 cloned yeast proteasomal genes nicely correspond to the 14 protein spots obtained after two-dimensional separation of the purified, dissociated yeast 20S proteasome [3]. Secondly, the 7 α-type and 7 β-type subunits found in yeast can easily be arranged into a structure with a twofold α7β7 symmetry as found in the ancestral archaebacterial proteasome [26]. The third reason to believe that the set of yeast proteasome subunits is complete comes from a comparison of these proteins with the primary structures of all so far known proteasomal subunits from other species: after multiple sequence alignment a dendrogram can be created which clearly shows 14 subgroups, 7 with α-type and 7 with β-type subunits, each of the subgroups containing a single yeast member (fig. 1). As supported by the gene disruption experiments in yeast, one can imagine that every yeast subunit occupies a defined place within the ring-like stacks of the proteasome, and that absence of individual subunits may disturb the assembly of the whole structure.

From the studies on the yeast 20S proteasome we further assume that a composition of 14 different subunit types occupying defined positions in a dimeric structure with C2-symmetry applies to all eukaryotic proteasomes.

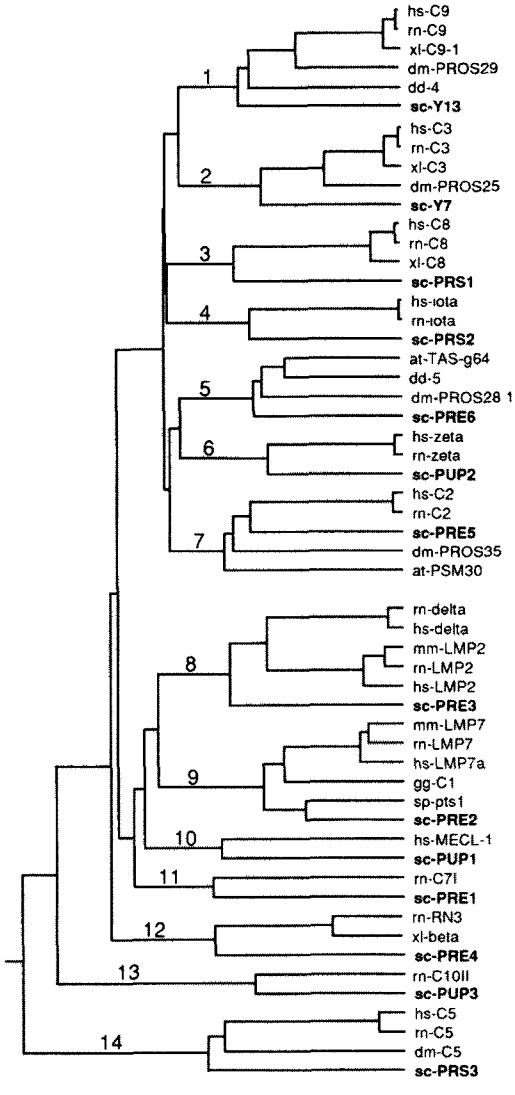

Fig. 1. Dendrogram showing the relationships among all known eukaryotic 20S proteasome protein sequences. The 14 *S. cerevisiae* proteasome subunits are shown in bold type. The 14 main branches are numbered. Subgroups 1–7 contain α-type members, subgroups 8–14 contain β-type members. In cases of subunits, for which slightly differing sequences have been reported, only one sequence is considered. Abbreviations for species are: at = *Arabidopsis thaliana;* dd = *Dictyostelium discoideum;* dm = *Drosophila melanogaster;* gg = *Gallus gallus;* hs = *Homo sapiens;* mm = *Mus musculus;* rn = *Rattus norvegicus;* sc = *S. cerevisiae,* sp = *S. pombe,* xl = *X. laevis.* The dendrogram was created by the CLUSTAL multiple sequence alignment program using the DNASTAR™ 'Lasergene' software.

However, development- or cell-type-specific variations in the subunit composition of 20S proteasomes from higher eukaryotes may modulate their activity and function: a well-studied example is the 'immuno proteasomes' [27], containing 2 specialized subunits encoded in the MHC class-II gene cluster, LMP2 and LMP7. These two γ-interferon-inducible subunits substitute for very similar constitutive subunits [27, 28] with housekeeping functions, thereby changing the proteasome's proteolytic activities [27, 29–31]. The incorporation of LMP2 and LMP7 into 'immunoproteasomes' enhances their ability to generate those types of peptides from cytosolic protein antigens, which have high affinity for binding to MHC class-I glycoproteins in the endoplasmic reticulum as a prerequisite for presentation at the cell surface. Therefore, in the dendrogram in figure 1, two branches are expected, each of which contains two different mammalian subunit types: the pairs LMP2/delta in one branch and LMP7/epsilon in the other. (Since only partial amino acid sequences are yet available for subunit epsilon, it is not included in figure 1.) The equivalents of these subunits in yeast are Pre2 and Pre3. These subunits have indeed been shown to be responsible for the proteolytic activities which are altered in 'immunoproteasomes' [5, 6].

The principle of interchangeability of strongly related proteasomal subunits may be a widespread mechanism in higher eukaryotes. This is indicated by the identification of slightly differing cDNAs reflecting either gene duplications [for example Fujii et al., 32] or alternative splicing [for example Silva Pereira et al., 33].

Additionally, posttranslational modifications of subunits are known, such as phosphorylation [34] and glycosylation [35], which may also have regulatory or modulating functions. Thus, a higher number than 14 protein spots generally observed in two-dimensional separations of mammalian, plant or insect proteasomes can be easily explained by such subunit modifications as well as by the existence of subunit isoforms. Thus, such data are not in conflict with the proposed model of a common 20S proteasome structure consisting of two sets of 14 subunit types.

The 26S Proteasome

A 26S protease which is able to degrade ubiquitinylated proteins in vitro has been purified from rabbit reticulocytes [36]. This protease complex is composed of a pool of at least 25 different subunits. Studies in several higher eukaryotes demonstrated that the 26S protease consists of the 20S proteasome as a core [37–40] and additional subunits attached at both ends of the 20S cylinder [41]. Recently, strong evidence appeared that this larger proteinase complex also exists in yeast. Under certain conditions a 26S protease complex can be purified from yeast, which exhibits peptide cleaving activities of the 20S proteasome but in addition is able to degrade ubiquitinylated proteins in vitro [Fischer et al., in preparation]. A functional indication for the existence of a 26S proteasome in yeast is provided by the fact that mutants with defects in peptide-cleaving activities of the 20S proteasome are also defective in the degradation of ubiquitinylated proteins in vivo, which are specific in vitro substrates of the 26S proteasome [3, 5, 7, 42, 43].

Using peptide sequences derived from purified 26S proteasomes three subunits of the human 26S complex have been characterized [44–46]. The protein sequences showed similarity or identity with formerly described transcriptional activator proteins of HIV gene expression [47, 48]. In the yeast *S. cerevisiae* two 26S proteasome specific genes *CIM3* and *CIM5* have been cloned by complementation

of temperature-sensitive mitotic cell cycle mutants [49]. Identity of Cim3 and Cim5 as 26S proteasome subunits has been shown biochemically by cross-reaction of Cim3- and Cim5-specific antibodies with corresponding subunits of the 26S proteasome purified from *Drosophila.* Besides cell cycle control, Cim3 and Cim5 also function in ubiquitin-dependent degradation of the short-lived protein Ub-Pro-β-gal [see the chapter: Short-Lived Proteins]. Both proteins Cim3 and Cim5 exhibit strong similarities with the human 26S proteasome subunits. Cim5 shows 70% identity with the human subunit S7/Mss1. The S7/Mss1 protein is also able to complement the lethality of a *CIM5* deletion mutant. *CIM3* is identical with *SUG1* which in a mutated form (*sug1-1)* has been found to suppress a transcriptional activation defect in a *gal4* deletion mutant which lacks the carboxyterminal transcriptional activation domain [50].

In the yeast *Schizosaccharomyces pombe* also a 26S subunit, *mts2*$^+$, has been cloned by complementation of mitotic cell cycle mutants [51]. The *mts2*$^+$ protein shows 75% identity with the human 26S subunit S4 and can be functionally replaced by the S4 protein.

The yeast and human 26S proteasome subunits can be assigned to a recently defined family of ATPases, which contain one or two copies of a strongly conserved region of 200 amino acids [52, 53]. Using polymerase chain reaction with oligonucleotides derived from highly conserved boxes within these ATPase-characteristic regions, 11 members of this ATPase family (*YTA1–YTA11)* have been cloned and sequenced in yeast [54]. Besides *YTA3* which is identical with *CIM5,* also *YTA1, YTA2* and *YTA5* have been proposed to code for yeast 26S proteasome subunits because of the strong sequence similarities of the encoded proteins. In fact a defective *yta1* allele is complemented by the human 26S subunit S6/Tbp7 [54].

Proteasome Functions

Proteasomes Are Essential to Life

Proteasomes fulfill essential functions in the yeast cell. In 13 cases chromosomal deletion of 1 of the 14 different yeast 20S proteasome subunits led to cell death. Spores derived from heterozygous diploids carrying a null mutation of 1 of several 20S proteasome genes were able to germinate, but stopped cell growth after 2–3 cell divisions [7, 17]. It is suggested from this result that chromosomal deletion of 1 of the 20S proteasome genes leads to disassembly of the 20S and 26S proteasome complex and therefore complete loss of all proteasome functions. Also 26S proteasome-specific genes serve essential functions. Chromosomal deletions of the yeast 26S proteasome subunits *CIM3, CIM5* [49], *YTA1* or *YTA2* [54] cause lethality.

Proteasomes Act in Stress-Dependent and Ubiquitin-Mediated Proteolytic Pathways

Yeast mutants bearing mutations in different subunits of the 20S proteasome leading to defects in peptide-cleaving activities have been used to demonstrate the in vivo function of the complex in stress-dependent and ubiquitin-mediated proteolytic pathways for the first time. Mutations affecting the proteasomal genes *PRE1* and *PRE2* lead to defects of the chymotrypsin-like activity [3, 5], whereas mutants of the proteasomal subunits *PRE3* and *PRE4* are defective in the PGPH activtiy of the complex [6, 7]. *Pre1-1* single and even more so *pre1-1 pre2-2* double mutants are sensitive to heat stress [3, 5]. They also show sensitivity to the arginine analog canavanine which leads to the formation of abnormal proteins (fig. 2). Under heat and canavanine stress conditions the mutants also accumulate ubiquitinylated proteins. It is suggested that heat and canavanine stress lead to the formation of large amounts of abnormal proteins

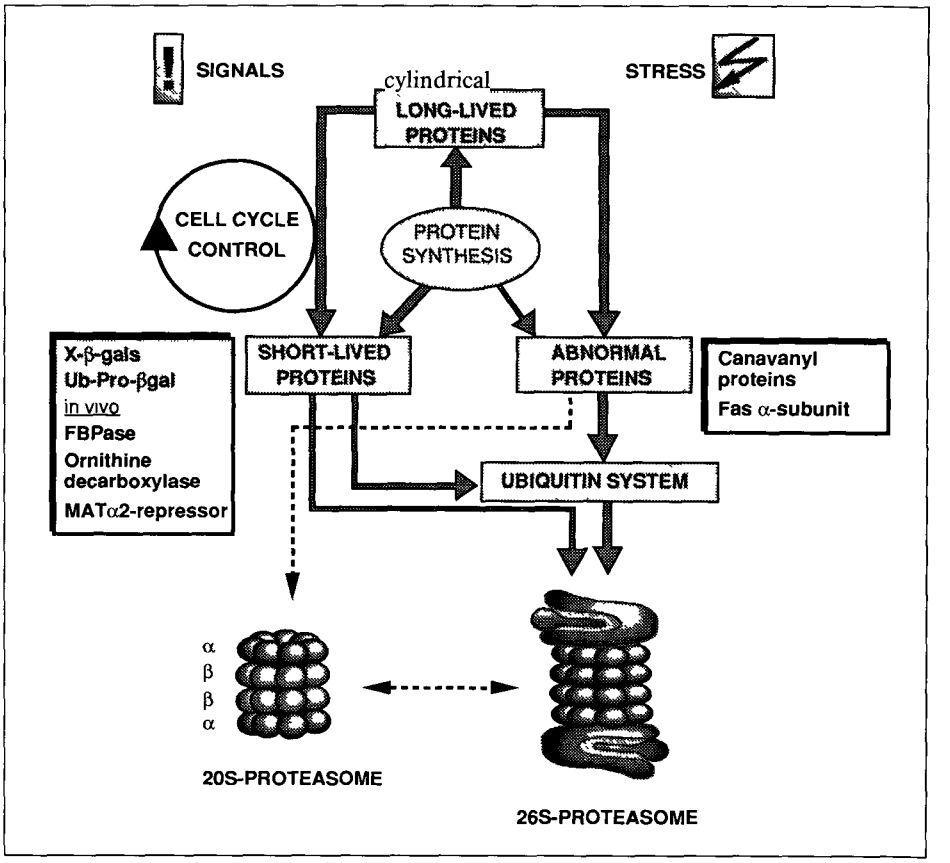

Fig. 2. Proteasomes fulfill central cellular functions in the eukaryote. The 20S proteasome is a cylindrical-shaped multienzyme complex which is also found as an integral core of the even larger 26S proteasome. Proteasomes are involved in the degradation of abnormal and short-lived proteins. In most cases prior to proteolysis proteins are marked for degradation by tagging systems, as is the ubiquitin system. Abnormal proteins are generated by synthesis errors or cellular stress. In combination with protein synthesis proteolysis functions by modulating the intracellular concentrations of constitutively short-lived proteins as well as proteins which become unstable due to specific signals or intracellular programs. By uncovering certain in vivo substrate proteins of the proteasome, studies in yeast demonstrated the function of this proteinase complex in metabolic adaptation, cell differentiation and cell cycle control.

which are ubiquitinylated. They are stabilized in the proteolysis-defective 20S proteasome mutants [3, 5]. Interestingly, in *pre4-1* single mutants which completely lack the PGPH activity of the 20S proteasome, no apparent defect in stress- and ubiquitin-dependent proteolysis has been observed [7]. However, in *pre4-1 pre1-1* double mutants the stress phenotypes, as well as the accumulation of ubiquitinylated proteins is strongly enhanced as compared to *pre1-1* single mutants [7]. The chymotrypsin-like activity seems to act as the major proteolytic activity in canavanine and heat stress-induced degradation pathways, whereas the PGPH activity is proposed to fulfill some rescue function.

Proteasomes Degrade Short-Lived N-End-Rule Substrates

The function of proteasomes in the degradation of ubiquitinylated proteins in vivo has been confirmed by use of the short-lived protein substrates of the N-end-rule pathway [42, 43]. Different versions of β-galactosidase (X-β-gal) with destabilizing amino acid residues at its aminoterminus constitute artificial short-lived proteins in yeast [55, 56], which are ubiquitinylated by the ubiquitin-conjugating enzyme Ubc2 and thereafter degraded [57] (fig. 2). The in wild-type short-lived N-end-rule substrates Arg-β-gal ($t^1/_2$ = 2 min) and Leu-β-gal ($t^1/_2$ = 3 min) are clearly stabilized in the yeast 20S proteasome mutants *pre1-1* and *pre2-2* with defective chymotrypsin-like activity [42, 43]. Stabilization of these short-lived proteins is even more enhanced in *pre1-1 pre2-2* double mutants [42]. These 20S proteasome mutants also stabilize the short-lived Ub-Pro-β-gal protein [42, 43] which is ubiquitinylated in an N-end-rule independent manner by the ubiquitin-conjugating enzymes Ubc4/Ubc5 [58]. Interestingly in the 26S proteasome mutants *cim3-1* and *cim5-1* Ub-Pro-β-gal is also stabilized whereas degradation of the N-end-rule substrate Leu-β-gal is not affected [49]. This indicates that 26S proteasomes are able to distinguish between substrate proteins derived from different ubiquitin-dependent pathways.

In vivo Substrates of the Proteasome

Initially the function of proteasomes in protein degradation was shown by accumulation of a bulk of undefined ubiquitinylated proteins under cellular stress conditions or by stabilization of artificial short-lived N-end-rule substrate proteins in 20S proteasome yeast mutants. However, the question about individual in vivo substrates of the proteasome remained.

Proteasomes Degrade Unassembled Proteins: The Fate of Free α-Subunits of the Yeast Fatty Acid Synthase

Yeast fatty acid synthase is an oligomeric enzyme complex composed of 6 α- and 6 β-subunits, which are encoded by the genes *FAS1* (β-subunit) and *FAS2* (α-subunit). The assembled fatty acid synthase complex is a stable protein with a $t^1/_2$ of at least 20 h [59]. In contrast, in a yeast strain lacking the fatty acid synthase β-subunits due to a chromosomal deletion of the *FAS1* gene, free Fas2 α-subunits are short lived ($t^1/_2$ = 1 h). The free α-subunits are rapidly degraded via the proteasome. This has been proven by the fact that 20S proteasome mutants *pre1-1* containing a *fas1* deletion allele clearly stabilize the Fas2 protein, as compared to *PRE1* wild-type cells [59]. The free α-subunit seems to be recognized as some sort of abnormal protein and is therefore rapidly degraded via the proteasomal pathway (fig. 2).

Proteasomes Degrade Regulated Metabolic Enzymes and Regulatory Proteins: Degradation of Fructose-1,6-Bisphosphatase

The function of proteasomes in the degradation of regulated and short-lived proteins has been demonstrated in the case of catabolite inactivation of fructose-1,6-bisphosphatase, which is a key enzyme in gluconeogenesis. Addition of glucose to yeast cells, which have been grown on a non-fermentable carbon source ($t^1/_2$ of fructose-1,6-bisphosphatase under derepression conditions is 90 h) induces inactivation of the enzyme by phosphorylation followed by rapid proteolytic degradation [60, 61]. In wild-type cells fructose-1,6-bisphosphatase is completely degraded within 1 h. In the *pre1-1* proteasome mutants fructose-1,6-bisphosphatase is strongly stabilized. In *pre1-1 pre2-1* double mutants defective in two subunits of the 20S proteasome,

degradation of fructose-1,6-bis-phosphatase is nearly absent [62]. Thus proteasomes regulate metabolic adaptation of cells upon changes in environmental conditions (fig. 2).

Ornithine Decarboxylase Is Degraded by the 26S Proteasome via a Ubiquitin-Independent Pathway. The involvement of 26S proteasomes also in ubiquitin-independent proteolytic pathways has been demonstrated for another metabolic enzyme, ornithine decarboxylase. This key enzyme of polyamine synthesis is subjected to rapid turnover in mammalian cells [63]. ATP-dependent degradation of ornithine decarboxylase is performed by an ubiquitin-independent mechanism which uses antizyme, a protein induced by polyamines, as a proteolytic accelerator [64]. Biochemical studies demonstrated that ornithine decarboxylase is degraded via the 26S proteasome [65, 66]. The function of proteasomes in the degradation of ornithine decarboxylase in vivo has also been proven using the 20S proteasome mutants defective in the chymotrypsin-like activity. Mouse ornithine decarboxylase expressed in *pre1-1* cells as well as the yeast ornithine decarboxylase are clearly stabilized in these proteasome mutants [67] (fig. 2).

Proteasomes Degrade a Transcriptional Regulator Protein, the MATα2 Repressor

The MATα2 repressor is a transcriptional regulator protein which is required for mating type differentiation in the yeast *S. cerevisiae* by repression of MATa-specific genes in haploid MATα cells and haploid-specific genes in diploid cells [68, 69]. The MATα2 repressor is a short-lived protein which is degraded with a $t^1/_2$ of 5 min at 30 °C [70]. The protein contains two independent destruction boxes and is degraded after ubiquitinylation via the ubiquitin-conjugating enzymes Ubc4/Ubc5 and also Ubc6/Ubc7 [70, 71]. The MATα2 repressor is stabilized in 20S proteasome mutants (*pre1-1* and *pre1-1 pre2-2)* [Richter-Ruoff et al., in preparation] which strongly indicates that proteasomes are involved in the degradation of this repressor protein (fig. 2). In a screen for mutants defective for the degradation of the MATα2 repressor, mutants of the de-ubiquitinating enzyme Doa4 were found. From this result it has been proposed that de-ubiquitinylation is coupled to the degradation of proteins by the 26S proteasome [72].

Proteasomes Function in Yeast Cell Cycle Control

Multiple kinase complexes formed by the association of different types of cyclins with a single kinase subunit (*Cdc28)* are thought to be crucial for cell cycle control in yeast [73]. Cln cyclins are required during G1 for commitment to a new cell cycle [74], whereas the B-type cyclins Clb5 and Clb6 are involved in cell entry into S phase [75, 76]. Clb1 to Clb5 B-type cyclins are needed during mitosis for assembly and function of the spindle apparatus [74, 76, 77]. The appearance and disappearance of particular kinase forms, which are thought to trigger key cell cycle events, is regulated by the synthesis and proteolytic degradation of specific cyclins during different phases of the cell cycle. Several lines of evidence exist that at least B-type cyclins are degraded by proteasomes via ubiquitin-dependent pathways. Studies of rapid mitotic degradation of B-type cyclins revealed an aminoterminally located highly conserved stretch of nine amino acids (designated as the 'destruction box') as well as ubiquitin modification prior to proteolysis [78].

The function of proteasomes in cell cycle control has been demonstrated by isolation of cell cycle mutants of the 26S and 20S proteasome of yeast. Two *S. cerevisiae* mutants *cim3-1* and *cim5-1* both containing a mutation in a 26S proteasome-specific subunit

which confer lethality to the G2/mitosis-specific *cdc28-1N* mutant have been isolated. In addition, the mutants exhibit a temperature-sensitive cell cycle phenotype [49]. At non-permissive temperature *cim3-1* and *cim5-1* mutant cells are arrested with replicated DNA and short intranuclear spindles characteristic of *S. cerevisiae* cells arrested in G2/metaphase. A corresponding G2/metaphase cell cycle arrest has been observed in *mts2* mutants of *S. pombe* which also contain a mutated 26S proteasome [51]. In both cases the cell cycle phenotypes seem to be due to proteolytic defects of the 26S proteasome. *Cim3-1* and *cim5-1* mutants clearly stabilize a short-lived cell cycle-independent ubiquitinylated protein (Ub-Pro-β-gal) [49] whereas *S. pombe mts2* mutants accumulate amounts of undefined ubiquitinylated proteins [51]. Further evidence for the involvment of proteasomes in cell cycle control has been obtained from studies of 20S proteasome mutants. Yeast cells with a proteolytically stable form of the cyclin Clb2 due to a deletion of the destruction box are arrested in the mitotic telophase [73, 79]. It has further been shown that overexpression of a single *CLB2* copy from the *GAL1* promoter is tolerated by the yeast cells while overexpression of four copies of *CLB2* is lethal [79]. However, in proteolysis-defective 20S proteasome *(pre1-1)* mutants the overexpression of even one copy of *CLB2* leads to the stop of cellular growth [80]. Plasmid mutagenesis with the 20S proteasomal gene *PRG1/PRE2* yielded temperature sensitive mutants which exhibit a mitotic cell cycle phenotype under nonpermissive temperatures [81]. Interestingly this phenotype is suppressed by chromosomal deletion of the *CLB2* gene [81]. Though not unequivocally proven, these studies in yeast suggest that proteasomes function in cell cycle control via the degradation of mitotic cyclins (fig. 2).

Concluding Remarks

Studies on the proteasome of the yeast *S. cerevisiae* reflect the great potential of this organism in the elucidation of basic eukaryotic cell functions. The ease with which yeast is amenable to biochemical, genetic and molecular biological studies greatly facilitated the detection and analysis of all 14 20S proteasome core subunits and made possible the discovery of the first in vivo functions of the enzyme complex. Many more in vivo functions will be discovered in the future. The examples we know today may guide us in the direction we have to search for proteasome functions, namely the removal of false and not properly folded proteins, the degradation of enzymes which regulate cellular metabolism and the degradation of regulatory proteins which regulate gene expression and the biological activity of proteins. As such the proteasome is a tool central for cellular life.

Acknowledgements

The authors were supported by grants of the German-Israeli Foundation for Scientific Research and Development, Jerusalem, the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt.

References

1 Achstetter T, Ehmann C, Osaki A, Wolf DH: Proteinase yscE, a new yeast peptidase. J Biol Chem 1984; 259:13344–13348.

2 Kleinschmidt JA, Escher C, Wolf DH: Proteinase yscE of yeast shows homology with the 20S cylinder particles of *Xenopus laevis*. FEBS Lett 1988;239:35–40.

3 Heinemeyer W, Kleinschmidt JA, Saidowsky J, Escher C, Wolf DH: Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: Mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival. EMBO J 1991;10:555–562.

4 Orlowski M, Cardozo C, Michaud C: Evidence for the presence of five distinct proteolytic components in the pituitary multicatalytic proteinase complex. Properties of two components cleaving bonds on the carboxyl side of branched chain and small neutral amino acids. Biochemistry 1993;32:1563–1572.

5 Heinemeyer W, Gruhler A, Mohrle V, Mahe Y, Wolf DH: *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. J Biol Chem 1993;268:5115–5120.

6 Enenkel C, Lehmann H, Kipper J, Guckel R, Hilt W, Wolf DH: *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. FEBS Lett 1994;341:193–196.

7 Hilt W, Enenkel C, Gruhler A, Singer T, Wolf DH: The *PRE4* gene codes for a subunit of the yeast proteasome necessary for peptidylglutamyl peptide-hydrolyzing activity: Mutations link the proteasome to stress-dependent and ubiquitin-dependent proteolysis. J Biol Chem 1993;268:3479–3486.

8 Friedman H, Goebel M, Snyder M: A homolog of the proteasome-related RING10 gene is essential for yeast cell growth. Gene 1992;122: 203–206.

9 Haffter P, Fox TD: Nucleotide sequence of *PUP1* encoding a putative proteasome subunit in *Saccharomyces cerevisiae*. Nucleic Acids Res 1991;19:5075.

10 Basile G, Aker M, Mortimer RK: Nucleotide sequence and transcriptional regulation of the yeast recombinational repair gene RAD51. Mol Cell Biol 1992;12:3235–3246.

11 Nishimura C, Tamura T, Akioka H, Tokunaga F, Tanaka K, Ichihara A: cDNA cloning of rat proteasome subunit RC10-II, assumed to be responsible for trypsin-like catalytic activity. FEBS Lett 1993;336:462–466.

12 Lee DH, Tanaka K, Tamura T, Chung CH, Ichihara A: *PRS3* encoding an essential subunit of yeast proteasomes homologous to mammalian proteasome subunit C5. Biochem Biophys Res Commun 1992; 182:452–460.

13 Fujiwara T, Tanaka K, Orino E, Yoshimura T, Kumatori A, Tamura T, Chung CH, Nakai T, Yamaguchi K, Shin S, Kakizuka A, Nakanishi S, Ichihara A: Proteasomes are essential for yeast proliferation. cDNA cloning and gene disruption of two major subunits. J Biol Chem 1990; 265:16604–16613.

14 Emori Y, Tsukahara T, Kawasaki H, Ishiura S, Sugita H, Suzuki K: Molecular cloning and functional analysis of three subunits of yeast proteasome. Mol Cell Biol 1991;11: 344–453.

15 Balzi E, Chen WN, Capieaux E, McCusker JH, Haber JE, Goffeau A: The suppressor gene *scl1*$^+$ of *Saccharomyces cerevisiae* is essential for growth (published erratum appears in Gene 1990;89:151). Gene 1989; 83:271–279.

16 Georgatsou E, Georgakopoulos T, Thireos G: Molecular cloning of an essential yeast gene encoding a proteasomal subunit. FEBS Lett 1992; 299:39–43.

17 Heinemeyer W, Tröndle N, Albrecht G, Wolf DH: *PRE5* and *PRE6*, the last missing genes encoding 20S proteasome subunits from yeast? Indication for a set of 14 different subunits in the eukaryotic proteasome core. Biochemistry 1994, in press.

18 Tanaka K, Yoshimura T, Kumatori A, Ichihara A, Ikai A, Nishigai M, Kameyama K, Takagi T: Proteasomes (multi-protease complexes) as 20 S ring-shaped particles in a variety of eukaryotic cells. J Biol Chem 1988;263:16209–16217.

19 Tanaka K, Tamura T, Kumatori A, Kwak TH, Chung CH, Ichihara A: Separation of yeast proteasome subunits. Immunoreactivity with antibodies against ATP-dependent protease Ti from *Escherichia coli*. Biochem Biophys Res Commun 1989; 164:1253–1261.

20 Lee LW, Moomaw CR, Orth K, McGuire MJ, De Martino GN, Slaughter CA: Relationships among the subunits of the high molecular weight proteinase, macropain (proteasome). Biochim Biophys Acta 1990;1037:178–185.

21 Lilley KS, Davison MD, Rivett AJ: N-terminal sequence similarities between components of the multicatalytic proteinase complex. FEBS Lett 1990;262:327–329.

22 Frentzel S, Kuhn HI, Gernold M, Gott P, Seelig A, Kloetzel PM: The major-histocompatibility-complex-encoded beta-type proteasome subunits LMP2 and LMP7. Evidence that LMP2 and LMP7 are synthesized as proproteins and that cellular levels of both mRNA and LMP-containing 20S proteasomes are differentially regulated. Eur J Biochem 1993;216:119–126.

23 Glynne R, Kerr LA, Mockridge I, Beck S, Kelly A, Trowsdale J: The major histocompatibility complex-encoded proteasome component LMP7 – alternative first exons and post-translational processing. Eur J Immunol 1993;23:860–866.

24 Zwickl P, Grziwa A, Puhler G, Dahlmann B, Lottspeich F, Baumeister W: Primary structure of the *Thermoplasma* proteasome and its implications for the structure, function, and evolution of the multicatalytic proteinase. Biochemistry 1992; 31:964–972.
25 Dick LR, Moomaw CR, Pramanik BC, De Martino GN, Slaughter CA: Identification and localization of a cysteinyl residue critical for the trypsin-like catalytic activity of the proteasome. Biochemistry 1992;31: 7347–7355.
26 Puhler G, Weinkauf S, Bachmann L, Muller S, Engel A, Hegerl R, Baumeister W: Subunit stoichiometry and three-dimensional arrangement in proteasomes from *Thermoplasma acidophilum* EMBO J 1992;11: 1607–1616.
27 Aki M, Shimbara N, Takashina M, Akiyama K, Kagawa S, Tamura T, Tanahashi N, Yoshimura T, Tanaka K, Ichihara A: Interferon-gamma induces different subunit organizations and functional diversity of proteasomes. J Biochem (Tokyo) 1994;115:257–269.
28 Früh K, Gossen M, Wang K, Bujard H, Peterson PA, Yang Y: Displacement of housekeeping proteasome subunits by MHC-encoded LMPs: A newly discovered mechanism for modulating the multicatalytic proteinase complex. EMBO J 1994;13: 3236–3244.
29 Driscoll J, Brown MG, Finley D, Monaco JJ: MHC-linked LMP gene products specifically alter peptidase activities of the proteasome. Nature 1993;365:262–264.
30 Gaczynska M, Rock KL, Goldberg AL: Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. Nature 1993;365:264–267.
31 Boes B, Hengel H, Ruppert T, Multhaup G, Koszinowski UH, Kloetzel P-M: Interferon-γ stimulation modulates the proteolytic activity and cleavage site preference of 20S mouse proteasomes. J Exp Med 1994;179:901–909.
32 Fujii G, Tashiro K, Emori Y, Saigo K, Shiokawa K: Molecular cloning of cDNAs for two *Xenopus* proteasome subunits and their expression in adult tissues. Biochim Biophys Acta 1993;1216:65–72.
33 Silva Pereira I, Bey F, Coux O, Scherrer K: Two mRNAs exist for the Hs PROS-30 gene encoding a component of human prosomes. Gene 1992;120:235–242.
34 Arrigo AP, Mehlen P: Hela cells proteasome interacts with leucine-rich polypeptides and contains a phosphorylated subunit. Biochem Biophys Res Commun 1993;194:1387–1393.
35 Tomek W, Adam G, Schmid HP: Prosomes, small cytoplasmic RNP particles, contain glycoproteins. FEBS Lett 1988;239:155–158.
36 Hough R, Pratt G, Rechsteiner M: Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates. J Biol Chem 1986;261:2400–2408.
37 Eytan E, Ganoth D, Armon T, Hershko A: ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated to ubiquitin. Proc Natl Acad Sci USA 1989;86:7751–7755.
38 Driscoll J, Goldberg AL: The proteasome (multicatalytic protease) is a component of the 1,500-kD proteolytic complex which degrades ubiquitin-conjugated proteins. J Biol Chem 1990;265:4789–4792.
39 Orino E, Tanaka K, Tamura T, Sone S, Ogura T, Ichihara A: ATP-dependent reversible association of proteasomes with multiple protein components to form 26S complexes that degrade ubiquitinated proteins in human HL-60 cells. FEBS Lett 1991;284:206–210.
40 Peters JM, Harris JR, Kleinschmidt JA: Ultrastructure of the approximately 26S complex containing the approximately 20S cylinder particle (multicatalytic proteinase/proteasome). Eur J Cell Biol 1991;56:422–432.
41 Peters JM, Cejka Z, Harris JR, Kleinschmidt JA, Baumeister W: Structural features of the 26-S proteasome complex. J Mol Biol 1993; 234:932–937.
42 Richter-Ruoff B, Heinemeyer W, Wolf DH: The proteasome/multicatalytic-multifunctional proteinase. In vivo function in the ubiquitin-dependent N-end rule pathway of protein degradation in eukaryotes. FEBS Lett 1992;302:192–196.
43 Seufert W, Jentsch S: In vivo function of the proteasome in the ubiquitin pathway. EMBO J 1992;11: 3077–3080.
44 Dubiel W, Ferrel K, Rechsteiner M: Tat-binding protein 7 is a subunit of the 26S protease. Biol Chem Hoppe Seyler 1994;375:237–240.
45 Dubiel W, Ferrell K, Pratt G, Rechsteiner M: Subunit-4 of the 26-S protease is a member of a novel eukaryotic ATPase family. J Biol Chem 1992;267:22699–22702.
46 Dubiel W, Pratt G, Ferrell K, Rechsteiner M: Purification of an 11 S regulator of the multicatalytic protease. J Biol Chem 1992;267: 22369–22377.
47 Nelbock P, Dillon PJ, Perkins A, Rosen CA: A cDNA for a protein that interacts with the human immunodeficiency virus Tat transactivator. Science 1990;248:1650–1653.
48 Shibuya H, Irie K, Ninomiya TJ, Goebl M, Taniguchi T, Matsumoto K: New human gene encoding a positive modulator of HIV Tat-mediated transactivation. Nature 1992; 357:700–702.
49 Ghislain M, Udvardy A, Mann C: *S. cerevisiae* 26S protease mutants arrest cell division in G2/metaphase. Nature 1993;366:358–362.
50 Swaffield JC, Bromberg JF, Johnston SA: Alterations in a yeast protein resembling HIV Tat-binding protein relieve requirement for an acidic activation domain in *GAL4*. Nature 1992;357:698–700.
51 Gordon C, McGurk G, Dillon P, Rosen C, Hastie ND: Defective mitosis due to a mutation in the gene for a fission yeast 26S protease subunit. Nature 1993;366:355–357.
52 Erdmann R, Wiebel FF, Flessau A, Rytka J, Beyer A, Frohlich KU, Kunau WH: *PAS1*, a yeast gene required for peroxisome biogenesis, encodes a member of a novel family of putative ATPases. Cell 1991;64: 499–510.
53 Frohlich KU, Fries HW, Rudiger M, Erdmann R, Botstein D, Mecke D: Yeast cell cycle protein Cdc48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. J Cell Biol 1991;114:443–453.

54 Schnall R, Mannhaupt G, Stucka R, Tauer R, Ehnle S, Schwarzlose C, Vetter I, Feldmann H: Identification of a set of yeast genes coding for a family of putative ATPases with high similarity to constituents of the 26S protease complex. Yeast 1994, in press.
55 Bachmair A, Finley D, Varshavsky A: In vivo half-life of a protein is a function of its amino-terminal residue. Science 1986;234:179–186.
56 Varshavsky A: The N-end rule. Cell 1992;69:725–735.
57 Dohmen RJ, Madura K, Bartel B, Varshavsky A: The N-end rule is mediated by the *UBC2(RAD6)* ubiquitin-conjugating enzyme. Proc Natl Acad Sci USA 1991;88:7351–7355.
58 Johnson ES, Bartel B, Seufert W, Varshavsky A: Ubiquitin as a degradation signal. EMBO J 1992;11: 497–505.
59 Egner R, Thumm M, Straub M, Simeon A, Schuller HJ, Wolf DH: Tracing intracellular proteolytic pathways. Proteolysis of fatty acid synthase and other cytoplasmic proteins in the yeast *Saccharomyces cerevisiae.* J Biol Chem 1993;268: 27269–27276.
60 Funayama S, Gancedo JM, Gancedo C: Turnover of yeast fructose-bisphosphatase in different metabolic conditions. Eur J Biochem 1980; 109:61–66.
61 Muller D, Holzer H: Regulation of fructose-1,6-bisphosphatase in yeast by phosphorylation/dephosphorylation. Biochem Biophys Res Commun 1981;103:926–933.
62 Schork SM, Bee G, Thumm M, Wolf DH: Site of catabolite inactivation. Nature 1994;369:283–284.
63 Pegg AE: Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J 1986;234:249–262.
64 Murakami Y, Tanaka K, Matsufuji S, Miyazaki Y, Hayashi S: Antizyme, a protein induced by polyamines, accelerates the degradation of ornithine decarboxylase in Chinese-hamster ovary-cell extracts. Biochem J 1992;283:661–664.
65 Murakami Y, Matsufuji S, Kameji T, Hayashi S, Igarashi K, Tamura T, Tanaka K, Ichihara A: Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. Nature 1992;360:597–599.
66 Bercovich Z, Kahana C: Involvement of the 20S-Proteasome in the degradation of ornithine decarboxylase. Eur J Biochem 1993;213:205–210.
67 Mamroud-Kidron E, Rosenberghasson Y, Rom E, Kahana C: The 20S proteasome mediates the degradation of mouse and yeast ornithine decarboxylase in yeast cells. FEBS Lett 1994;337:239–242.
68 Nasmyth K, Shore D: Transcriptional regulation in the yeast life cycle. Science 1987;237:1162–1170.
69 Herskowitz I: Life cycle of the budding yeast *Saccharomyces cerevisiae.* Microbiol Rev 1988;52:536–553.
70 Hochstrasser M, Varshavsky A: In vivo degradation of a transcriptional regulator: the yeast alpha 2 repressor. Cell 1990;61:697–708.
71 Chen P, Jentsch S, Hochstrasser M: Multiple ubiquitin-conjugating enzymes. Cell 1993;74:357–369.
72 Papa FR, Hochstrasser M: The yeast *DOA4* gene encodes a deubiquitinating enzyme related to a product of the human tre-2 oncogene. Nature 1993;366:313–319.
73 Nasmyth K: Control of the yeast cell cycle by the Cdc28 protein kinase. Curr Opin Cell Biol 1993;5:166–179.
74 Richardson HE, Wittenberg C, Cross F, Reed SI: An essential G1 function for cyclin-like proteins in yeast. Cell 1989;59:1127–1133.
75 Epstein CB, Cross FR: CLB5: A novel B cyclin from budding yeast with a role in S phase. Genes Dev 1992;6:1695–1706.
76 Schwob E, Nasmyth K: *CLB5* and *CLB6,* a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae.* Genes Dev 1993;7:1160–1175.
77 Fitch I, Dahmann C, Surana U, Amon A, Nasmyth K, Goetsch L, Byers B, Futcher B: Characterization of four B-type cyclin genes of the budding yeast *Saccharomyces cerevisiae* Mol Biol Cell 1992;3: 805–818.
78 Glotzer M, Murray AW, Kirschner MW: Cyclin is degraded by the ubiquitin pathway. Nature 1991;349: 132–138.
79 Surana U, Amon A, Dowzer C, McGrew J, Byers B, Nasmyth K: Destruction of the *CDC28/CLB* mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. EMBO J 1993;12: 1969–1978.
80 Richter-Ruoff B, Wolf DH: Proteasome and cell cycle. Evidence for a regulatory role of the protease on mitotic cyclins in yeast. FEBS Lett 1993;336:34–36.
81 Friedman H, Snyder M: Mutations in *PRG1,* a yeast proteasome-related gene, cause defects in nuclear division and are suppressed by deletion of a mitotic cyclin gene. Proc Natl Acad Sci USA 1994;91:2031–2035.