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In vivo function of the proteasome in the ubiquitin pathway

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A major eukaryotic proteolytic system is known to require the covalent attachment of ubiquitin to substrates prior to their degradation, yet the proteinase involved remains poorly defined. The proteasome, a large conserved multi-subunit protein complex of the cytosol and the nucleus, has been implicated in a variety of cellular functions. It is shown here that a yeast mutant with a defective proteasome fails to degrade proteins which are subject to ubiquitin-dependent proteolysis in wild-type cells. Thus, the proteasome is part of the ubiquitin system and mediates the degradation of ubiquitin–protein conjugates *in vivo*.

Key words: proteasome/protein degradation/ubiquitin system/yeast

Introduction

A major pathway for the selective degradation of proteins in eukaryotes requires the modification of proteolytic substrates by the covalent attachment of ubiquitin, a small (8.5 kDa), abundant and highly conserved cellular protein (reviewed in Finley and Chau, 1991; Jentsch, 1992). This pathway is highly selective and is thought to be largely regulated at the level of ubiquitin conjugation. Protein degradation by the ubiquitin system serves to eliminate damaged or otherwise abnormal proteins; a function which is essential for cell viability under stress conditions (Finley *et al.*, 1987; Seufert and Jentsch, 1990). Moreover, the ubiquitin system controls the half-lives of certain regulatory proteins. Targets include transcriptional regulators (Hochstrasser *et al.*, 1991), the tumor suppressor protein p53 (Scheffner *et al.*, 1990) and the cell cycle regulator cyclin (Glotzer *et al.*, 1991).

The transfer of ubiquitin to protein substrates is catalyzed by members of a large family of ubiquitin-conjugating (E2) enzymes (reviewed in Jentsch *et al.*, 1990) following an initial activation step by ubiquitin-activating (E1) enzyme. Target proteins appear to bear specific signals that are either recognized directly by E2 enzymes or by associated substrate-recognition proteins, known as E3 proteins. Conjugated ubiquitin can be a substrate for further ubiquitinations, leading to the formation of protein conjugates with isopeptide-linked multi-ubiquitin chains (Chau *et al.*, 1989). Multi-ubiquitination of substrates seems to facilitate the recognition by the ubiquitin conjugate-degrading protease. Whereas much has been learned about the mechanisms and functions of ubiquitin–protein conjugation in recent years, the protase degrading ubiquitin–protein conjugates *in vivo* remains to be defined. In previous studies, a 26 S protein complex was identified as being capable of degrading such conjugates *in vitro* (Hough *et al.*, 1986, 1987; Waxman *et al.*, 1987). The relatedness of this protein complex to the smaller, previously characterized 20 S proteasome is a matter under debate (Ganoth *et al.*, 1988; Eytan *et al.*, 1989; Matthews *et al.*, 1989; Driscoll and Goldberg, 1990; Seelig *et al.*, 1991).

The 20 S proteasome (reviewed in Rivett, 1989; Orlowski, 1990) is a large hollow cylindrical particle composed of four stacked rings that each contain six polypeptides. The subunit proteins whose genes have been cloned so far are related in sequence to each other but are unrelated to any known proteases (Emori *et al.*, 1991). The proteasome is present in both the cytosol and the nucleus of eukaryotic cells (Arrigo *et al.*, 1988; Haass *et al.*, 1989). Different functions have been attributed to this particle including proteolysis (Waxman *et al.*, 1985; Falkenburg *et al.*, 1988; Arrigo *et al.*, 1988), rRNA degradation (Tsukahara *et al.*, 1989), pre-tRNA processing (Castano *et al.*, 1986) and control of mRNA translation (Schmid *et al.*, 1984). *In vitro*, the proteasome exhibits three distinct endopeptidase activities, trypsin-like, chymotrypsin-like and peptidyl-glutamyl peptide hydrolyzing activity, cleaving bonds on the carboxyl side of basic, neutral/hydrophobic and acidic amino acids respectively (Tanaka *et al.*, 1988).

The *Saccharomyces cerevisiae* proteinase yscE (Achstetter *et al.*, 1984) was shown to be the yeast homolog of the vertebrate proteasome (Kleinschmitt *et al.*, 1988). Several genes encoding subunits of the yeast proteasome have been cloned (Fujiwara *et al.*, 1990; Emori *et al.*, 1991; Heinemeyer *et al.*, 1991). Extensive sequence similarities to *Drosophila* and mammalian proteasome subunits exist (Haass *et al.*, 1989; Tanaka *et al.*, 1990; Kumatori *et al.*, 1990). The majority of the yeast proteasomal genes are essential for cell viability. A mutation in one of these essential genes, *PRE1*, was isolated that affected the chymotrypsin-like activity of the proteasome (Heinemeyer *et al.*, 1991). This mutation causes a slow growth phenotype (W.Seufert and S.Jentsch, unpublished data) and leads to defects in cellular protein degradation, inviability of mutant cells at elevated temperature and hypersensitivity to an amino acid analog (Heinemeyer *et al.*, 1991). Similar phenotypes were observed with yeast mutants deficient in UBC4 and UBC5 ubiquitin-conjugating enzymes. These mutants are defective in a major proteolytic pathway (Seufert and Jentsch, 1990).

We show here that certain short-lived proteins, known to be degraded by the ubiquitin system in wild-type cells, are metabolically stabilized in *pre1* mutants, indicating that the proteasome is an integral part of the ubiquitin system *in vivo*.

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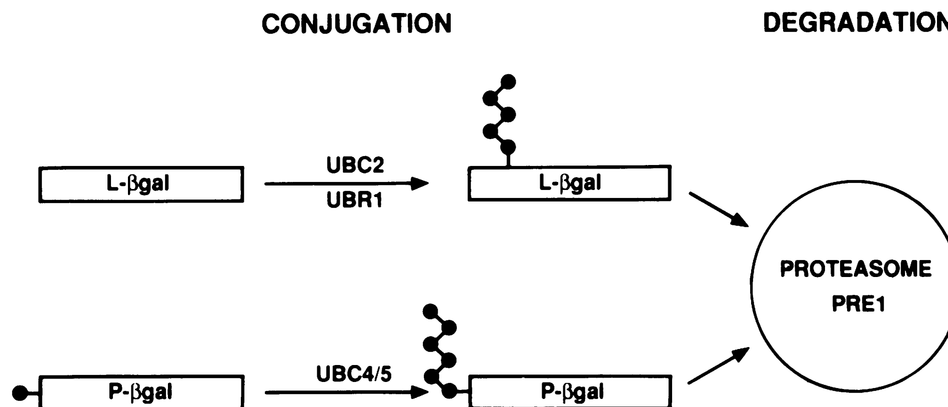

Fig. 1. Scheme of two different ubiquitin-dependent targeting pathways for the degradation by the proteasome. PRE1 is one of the proteasomal subunits. **Top**, L−βgal is a substrate of the N-end rule pathway. A multi-ubiquitin chain (symbolized by a chain of filled circles) is conjugated to a specific lysine residue of the substrate (open box), in a reaction depending on the recognition protein UBR1 and the UBC2 ubiquitin-conjugating enzyme. **Bottom**, in the case of ubiquitin-P−βgal, the ubiquitin moiety of the fusion (lollipop symbol on the left) is the target for the attachment of a multi-ubiquitin chain. This reaction is mediated by the UBC4 and UBC5 ubiquitin-conjugating enzymes (see text for details).

Results and discussion

Construction of a proteasome mutant strain expressing substrates of the ubiquitin system

To generate congenic wild-type and *pre1* mutant strains, the *PRE1* gene was disrupted in the homozygous diploid strain DF5 (for details see Materials and methods). Tetrad analysis after sporulation of heterozygote transformants indicated that cells carrying the *PRE1* gene disruption are inviable. Viability of *pre1* disruptants was rescued either by the wild-type *PRE1* gene (strain YWO71) or the mutant *pre1-1* allele (strain YWO74) on an ARS-CEN plasmid. These strains, isogenic except for the *PRE1* gene, were transformed with plasmids expressing substrates of the ubiquitin pathway.

The test proteins used are known to be targeted for degradation by two mechanistically different ubiquitin-dependent pathways. One pathway acts on proteins bearing a signal consisting of an amino-terminal residue of a destabilizing class according to the N-end rule (Bachmair *et al.*, 1986). This pathway employs the substrate recognition protein UBR1 (Bartel *et al.*, 1990) and the UBC2 ubiquitin-conjugating enzyme (Dohmen *et al.*, 1991) and attaches a multi-ubiquitin chain to a specific lysine residue of the substrate (Chau *et al.*, 1989; Figure 1). The second pathway acts on certain ubiquitin fusion proteins and utilizes ubiquitin as a signal for further ubiquitinations (Johnson *et al.*, 1992; Figure 1). In this case a multi-ubiquitin chain is confined to a lysine residue of the ubiquitin moiety of the fusion protein. This reaction involves UBC4 and UBC5 ubiquitin-conjugating enzymes.

Specifically engineered ubiquitin−β-galactosidase (βgal) fusion proteins are substrates for both targeting pathways (Bachmair *et al.*, 1986). Ubiquitin is rapidly removed from ubiquitin-X−βgal fusion proteins (X stands for the first C-terminal residue of the ubiquitin moiety of the fusion) by the activities of ubiquitin C-terminal hydrolases *in vivo*, thus exposing the N-terminal residue of the remaining part for recognition by the N-end rule pathway. However, ubiquitin-proline (P)−βgal is only very slowly processed by the hydrolase. In wild-type cells methionine (M)−βgal is a stable protein, whereas both leucine (L)−βgal and ubiquitin-P−βgal have very short half-lives (Bachmair *et al.*, 1986).

Table I. Levels of βgal activity in yeast wild-type (YWO71) and congenic *pre1* mutant (YWO74) strains expressing ubiquitin-X−βgal fusion proteins

	βgal activity[a]	
	Wild-type	*pre1*
M−βgal	190	160
L−βgal	5	170
Ubiquitin-P−βgal	14	180

[a]βgal activity of whole cells is given in Miller units and was measured as described (Reynolds and Lundblad, 1989). Values are the means of at least three independent measurements, deviations were <15%.

Stabilization of test proteins in a proteasome mutant

To assess a function of the proteasome in ubiquitin-dependent protein degradation *in vivo*, we analyzed the metabolic stability of the short-lived βgal derivatives, described above, in the *pre1* mutant strain. As expected, steady-state levels of βgal activity of the short-lived L−βgal and ubiquitin-P−βgal are low compared to the level of the stable M−βgal in the wild-type strain (Table I). In the *pre1* mutant strain, however, βgal activities of both test proteins approached the M−βgal value suggesting a metabolic stabilization of these proteins in the proteasome mutant (Table I).

Protein half-lives were directly determined by pulse−chase analyses. Cells were labeled briefly with [^{35}S]methionine and protein extracts were either prepared immediately or following a chase period. Lysates were subjected to immunoprecipitation using a monoclonal antibody specific for βgal. The sizes of the detected proteins (Figure 2) confirmed that in both wild-type and *pre1* mutant cells, ubiquitin is rapidly processed by the activities of ubiquitin C-terminal hydrolases from ubiquitin-M−βgal and ubiquitin-L−βgal fusion proteins, but not from ubiquitin-P−βgal. M−βgal was a stable protein both in the wild-type and *pre1* mutant strain. Degradation of L−βgal and ubiquitin-P−βgal occurred rapidly in wild-type cells and was accompanied by a multi-ubiquitination of the proteolytic substrates, as indicated by a ladder of slowly migrating bands above the substrate proteins in an SDS−polyacrylamide gel (Figure 2). These bands were absent in the case of M−βgal

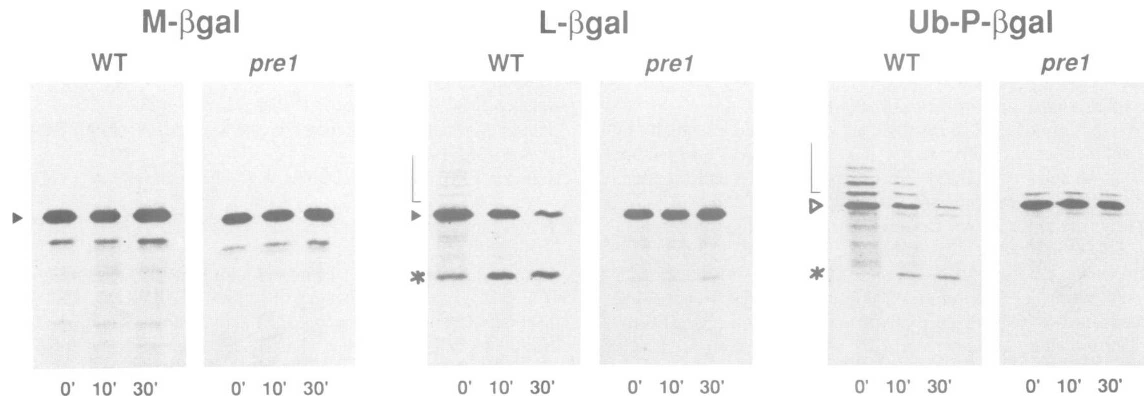

Fig. 2. Metabolic stability of proteolytic substrates of the ubiquitin pathway in wild-type (WT) and *pre1* mutant cells. Half-lives of M−βgal, L−βgal and ubiquitin (Ub)-P−βgal were analyzed by pulse−chase experiments as described (Bartel *et al.*, 1990). Wild-type (YWO71) and congenic *pre1* mutant (YWO74) strains expressing Ub-X−βgal proteins were labeled with [^{35}S]methionine for 3 min, followed by a chase for 0, 10 or 30 min, protein extraction, immunoprecipitation using a monoclonal antibody specific for βgal, SDS−PAGE and fluorography. Filled triangles indicate the positions of M−βgal and L−βgal, the open triangle that of the larger Ub-P−βgal. A ladder of multiply ubiquitinated βgal proteins is indicated by half-open brackets. Asterisks denote an ~90 kDa long-lived cleavage product of βgal.

which is not a substrate for ubiquitination. In *pre1* mutant cells, however, both L−βgal and ubiquitin-P−βgal were apparently stable proteins. We observed no significant turnover during the 30 min chase period. Thus, a mutation in a single subunit of the yeast 20 S proteasome has a dramatic effect on the metabolic stability of proteins normally degraded by the ubiquitin pathway in wild-type cells. Since the two test proteins used differ significantly in their targeting pathways (Figure 1), this strongly suggests that, in general, degradation of ubiquitin−protein conjugates is mediated by the proteasome *in vivo*.

Ubiquitination of proteolytic substrates in a proteasome mutant

Surprisingly, the block in degradation of the test proteins in *pre1* mutants did not result in an accumulation of multiply ubiquitinated species. Instead, the formation of a significant steady-state level of such conjugates appeared to be inhibited in the mutant (Figure 2). This unexpected observation may suggest that the proteasome also participates in ubiquitin−protein conjugation, possibly by a direct coupling of substrate conjugation and degradation. Alternatively, ubiquitin−protein conjugates which are not degraded by the proteasome are rapidly de-conjugated by the activities of ubiquitin C-terminal hydrolases.

Possible function of the ubiquitin/proteasome system in protein processing pathways

In wild-type cells ubiquitin-dependent degradation of βgal test proteins leads to the generation of a relatively stable protein fragment of ~90 kDa, corresponding to the C-terminal part of βgal (Bachmair *et al.*, 1986; Figure 2, band labeled with an asterisk). Intriguingly, this product is not generated in the *pre1* mutant. This observation suggests a novel function of the ubiquitin/proteasome system in cellular protein processing pathways. The proteasome was previously thought to degrade proteins to acid-soluble peptides and free amino acids only (reviewed in Goldberg, 1992). One possible substrate for a proteasome-mediated processing activity might be the p105 precursor of the p50 subunit of the transcription factor NF-κB which is processed to the mature form by an ATP-dependent reaction with characteristics of a ubiquitin/proteasome system (Fan and Maniatis, 1991).

Recently, proteasomal subunits have been shown to be encoded in the class II region of the major histocompatibility complex (MHC), leading to the speculation that the proteasome might be involved in antigen presentation pathways (Brown *et al.*, 1991; Glynne *et al.*, 1991; Ortiz-Navarrete *et al.*, 1991; Martinez and Monaco, 1991). The results presented here show that the proteasome is an essential and integral part of the ubiquitin-dependent pathways for protein degradation and processing. Together, these observations raise the possibility that the ubiquitin system might also be part of an antigen presentation pathway. It remains to be shown if other ubiquitin-related functions (reviewed in Jentsch, 1992) such as DNA repair, cell cycle progression, peroxisome biogenesis (F.F.Wiebel and W.-H.Kunau, personal communication) and transcriptional control are mediated by a proteasome-dependent degradation pathway.

Materials and methods

Construction of yeast strains and plasmids

Standard protocols were followed for growth of yeast strains, yeast transformation by the lithium acetate method, sporulation, tetrad dissection, preparation of total yeast DNA, and for the construction of plasmids (Ausubel *et al.*, 1989).

The *S.cerevisiae* strains used in this work are derived from the homozygous diploid strain DF5 (*MAT***a**/*MATα*, *his3-Δ200/his3-Δ200, leu2-3,2-112/leu2-3,2-112, lys2-801/lys2-801, trp1-1(am)/trp1-1(am), ura3-52/ura3-52*; Finley *et al.*, 1987). The *PRE1* gene was disrupted by inserting the *TRP1* marker at the unique *Sac*I restriction site (codon 74 of *PRE1*). Haploid cells carrying this *PRE1* gene disruption are inviable as described for the deletion of the *PRE1* gene (Heinemeyer *et al.*, 1991). Viability of *pre1* disruptants was rescued either by an *ARS1 CEN4 HIS3* plasmid (pSE362) carrying a 1.15 kb *Eco*RI−*Sca*I fragment encoding either wild-type *PRE1* (strain YWO71, *MATα*) or the mutant *pre1-1* allele (strain YWO74, *MATα*). For cloning the *pre1* mutant allele, DNA was prepared from the *pre1* strain BR2 (supplied by D.H.Wolf) and amplified by a polymerase chain reaction with 5′-primer WS49 (GAATTCCTTTTGACAGGTTC) and 3′-primer WS48 (AGTACTATCGTAGCCCTAC).

Determination of βgal activity

Strains YWO71 and YWO74 transformed with ubiquitin-X−βgal expressing plasmids (Bachmair *et al.*, 1986) and growing exponentially at 30°C in Sgal

medium (0.67% yeast nitrogen base, 2% galactose, supplemented with 30 μg/ml leucine and 30 μg/ml lysine) were analyzed for whole cell βgal activity by the ONPG assay (Reynolds and Lundblad, 1989).

Pulse–chase analysis and immunoprecipitation

The metabolic stability of βgal derivatives was determined essentially as described (Bachmair *et al.*, 1986; Bartel *et al.*, 1990). A 10 ml culture (OD_{600} = 0.2) of cells (see above) was harvested by centrifugation, resuspended in 0.6 ml Sgal medium and labeled with 150 μCi [^{35}S]-methionine (Amersham) for 3 min. Cells were harvested either immediately or following a chase in Sgal medium containing 0.5 mg/ml cycloheximide and 40 μg/ml methionine for 10 or 30 min. Cells were resuspended in 0.15 ml of cold buffer A (Bartel *et al.*, 1990) and disrupted by vortexing with glass beads for 3 min. Buffer A (0.6 ml) was added and extracts were cleared by centrifugation. As determined by TCA precipitation extracts routinely contained equal amounts of labeled proteins (deviations were <20%). After pre-clearing with protein A–Sepharose beads (Pharmacia) extracts were incubated with 1 μg of a mouse anti-βgal monoclonal antibody (catalogue no. Z3781, Promega) at 4°C for 3 h. Fifty microliters of a protein A–Sepharose suspension was added and after 1 h beads were collected by centrifugation, washed three times wtih 200 μl buffer A plus 0.1% SDS, resuspended in sample buffer (3% SDS, 50 mM DTT, 12% glycerol; 5 mM EDTA, 125 mM Tris–HCl, pH 6.8) and heated at 100°C for 3 min. Proteins were separated on 9% SDS–polyacrylamide gels followed by fluorography.

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