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Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome

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A family of ATPases resides within the regulatory particle of the proteasome. These proteins (Rpt1–Rpt6) have been proposed to mediate substrate unfolding, which may be required for translocation of substrates through the channel that leads from the regulatory particle into the proteolytic core particle. To analyze the role of ATP hydrolysis in protein breakdown at the level of the individual ATPase, we have introduced equivalent site-directed mutations into the ATP-binding motif of each *RPT* gene. Non-conservative substitutions of the active-site lysine were lethal in four of six cases, and conferred a strong growth defect in two cases. Thus, the ATPases are not functionally redundant, despite their multiplicity and sequence similarity. Degradation of a specific substrate can be inhibited by ATP-binding-site substitutions in many of the Rpt proteins, indicating that they co-operate in the degradation of individual substrates. The phenotypic defects of the different *rpt* mutants were strikingly varied. The most divergent phenotype was that of the *rpt1* mutant, which was strongly growth defective despite showing no general defect in protein turnover. In addition, *rpt1* was unique among the *rpt* mutants in displaying a G_1 cell-cycle defect. Proteasomes purified from an *rpt2* mutant showed a dramatic inhibition of peptidase activity, suggesting a defect in gating of the proteasome channel. In summary, ATP promotes protein breakdown by the proteasome through multiple mechanisms, as reflected by the diverse phenotypes of the *rpt* mutants.

Keywords: ATPase/proteasome/protein degradation/ubiquitin/yeast

Introduction

The ubiquitin–proteasome pathway is a major mediator of post-translational control in eukaryotes, which functions in the control of cell proliferation, the cell cycle and other processes (Hershko and Ciechanover, 1998). Conjugation of ubiquitin to proteolytic substrates such as cyclins and p53 targets the substrates for degradation by the proteasome. The proteasome (Coux *et al*., 1996; Larsen and Finley, 1997; Baumeister *et al*., 1998) is highly conserved throughout evolution. In yeast, it contains at least 32 subunits, distributed into two subcomplexes, the core particle and the regulatory particle (Fujimuro *et al*., 1998; Glickman *et al*., 1998a). In the cylindrical core particle, the proteolytic active sites face an internal cavity that can be accessed only through narrow channels found at the two ends of the cylinder (Löwe *et al*., 1995). These structural features imply that a key step in degradation is translocation of the substrate into the lumen of the core particle and that, due to the dimensions of the channel, unfolding of the protein substrate must precede translocation and degradation.

The channels open out into the regulatory particle (also known as PA700, the 19S cap complex and the μ complex), which is thought to mediate the putative early steps in degradation, such as substrate recognition (Pickart, 1997; Beal *et al*., 1998; Fu *et al*., 1998; Young *et al*., 1998). That the regulatory particle may play an active role in the degradation process is suggested by the fact that six of its subunits have sequence features of ATPases (Dubiel *et al*., 1992, 1993; DeMartino *et al*., 1994; Rubin *et al*., 1996; Richmond *et al*., 1997; Glickman *et al*., 1998a). ATPase activity has been demonstrated biochemically for several family members (Lucero *et al*., 1995; Berezutskaya and Bagchi, 1997; Fraser *et al*., 1997). Insofar as all six of the ATPases appear to reside within a single regulatory particle (Glickman *et al*., 1998a), the proteasome is among the most complex ATPase assemblies to be described.

ATP hydrolysis is strictly required for protein breakdown by the proteasome (Hershko *et al*., 1984; DeMartino *et al*., 1994; Hoffman and Rechsteiner, 1994). One hypothesis is that the energy requirement may reflect a role of the proteasomal ATPases in substrate unfolding. In this model, the role of ATP in the proteasome would be analogous to its role in the function of the ATPase ring complexes known as chaperonins, whose function is to assist in protein folding. In the chaperonins, ATP hydrolysis is used to drive large-scale structural transitions between states of high and low substrate-binding affinity, with an apparently concerted motion of the subunits (Fenton and Horwich, 1997; Ditzel *et al*., 1998). A second possible function for the proteasomal ATPases was suggested by the crystal structure of the yeast core particle. The ends of the cylinder were found in a closed state (Groll *et al*., 1997), suggesting that the proteasome channel is gated. The ATPases are candidates for mediating this gating. Finally, ATP is required for assembly of the proteasome from isolated regulatory and core particles (Armon *et al*., 1990; DeMartino *et al*., 1994; Hoffman and Rechsteiner, 1994).

The proteasomal ATPases of yeast are encoded by *RPT1–RPT6*, a distinct gene family (Figure 1) (Glickman *et al*., 1998a). Each of the Rpt proteins is highly conserved evolutionarily: sequence identities range from 66 to 76% between yeast and human homologs (Glickman *et al*.,

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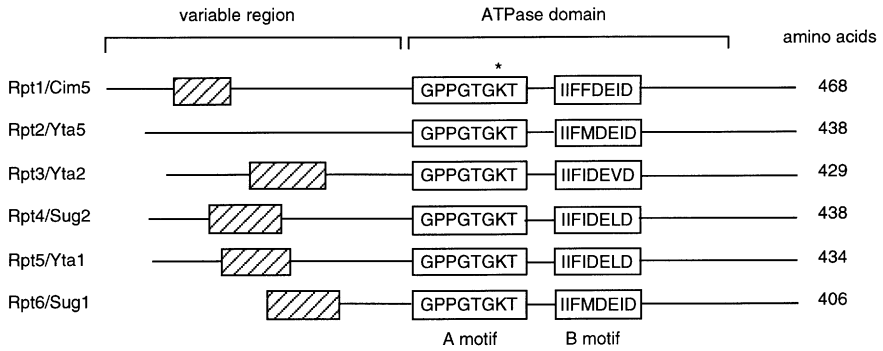

Fig. 1. Structural alignment of yeast Rpt proteins. Rpt proteins contain a conserved ATPase module which contains the A and B motifs that form the predicted ATP-binding domain (Walker *et al*., 1982; Gorbalenya and Koonin, 1989; Gorbalenya *et al*., 1989; Mian, 1993). An asterisk indicates the invariant Lys residues that were substituted with Arg or Ser. The N-termini of five of the proteins may contain a coiled-coil domain (hatched box), as predicted by the COILS2 program (Lupas *et al*., 1991; A.Lupas, personal communication).

1998a). While the *RPT* genes are essential for vegetative growth (Ghislain *et al*., 1993; Gordon *et al*., 1993; Schnall *et al*., 1994; Russell *et al*., 1996; Seeger *et al*., 1996), it is not clear whether this reflects a functional requirement for each ATPase or simply that in the complete absence of an Rpt subunit the proteasome fails to assemble. Point mutations have been isolated in many of the *RPT* genes, but there has been little biochemical analysis of these mutants, and in most cases the nature of the mutation has not been identified (Swaffield *et al*., 1992; Ghislain *et al*., 1993; Gordon *et al*., 1993; Campbell *et al*., 1994; Russell *et al*., 1996; Seeger *et al*., 1996; Gerlinger *et al*., 1997; McDonald and Byers, 1997).

The Rpt proteins belong to the Walker family of ATPases, as defined by specific ATP-binding motifs, including the Walker A motif. The invariant lysine residue of the A motif (Figure 1) characteristically interacts with the phosphate groups of ATP (Walker *et al*., 1982). Each of the proteasomal ATPases is identical in sequence throughout the A motif (Figure 1). Site-directed mutagenesis within the A motif region is thus uniquely suitable for providing a controlled functional comparison of the proteasomal ATPases. In this report, we describe the *in vivo* and *in vitro* consequences of such mutations.

The results described below demonstrate an unexpected diversity of function among the proteasomal ATPases, evidenced most dramatically by qualitative differences among them in both *in vivo* and *in vitro* phenotypes. Given the multiplicity of the proteasomal ATPases, it is remarkable that they display such a high degree of non-redundancy. In contrast, for the simple ATP-dependent proteases of prokaryotes, such as ClpAP and HslVU, the subunits of any given complex are apparently functionally equivalent (see reviews by Coux *et al*., 1996; Gottesman *et al*., 1997). In these proteases, the ATPase subunits form a homomeric ring complex that binds to the symmetrical ends of the cylindrical core particle (Gottesman *et al*., 1997; Larsen and Finley, 1997). By analogy, the Rpt proteins have been postulated to form a ring complex (Baumeister *et al*., 1998; Glickman *et al*., 1998a) in contact with the core particle, with substrates passing to the core particle through the center of the ATPase ring. Consistent with this model, the six ATPases, together with the Rpn1/Nas1 and Rpn2/Sen3 subunits, constitute a domain of the regulatory particle that directly contacts the core particle (Glickman *et al*., 1998b). That the functional equivalence typical of subunits of a ring complex was not observed in our genetic studies of the Rpt proteins may reflect not only the intrinsic functional properties of each ATPase but also that the proteasomal ATPases are contained within a larger, distinctly asymmetric structure, the proteasomal regulatory particle.

Results

Diverse phenotypes of Walker A motif mutants

The Walker ATPase family is characterized by two conserved sequence elements, the A and B motifs. Substitution of the invariant lysine of the A motif generally results in complete or partial inhibition of ATP binding and ATP hydrolysis (Sung *et al*., 1988), with the severity of the effect depending on whether the substitution is conservative or non-conservative. By substituting the same residue in each Rpt protein, we have generated a panel of mutants that are equivalent to one another and whose phenotypic differences are therefore expected to reflect accurately functional differences among these ATPases. In addition, the mutant phenotypes are expected to result from specific defects in ATP binding and hydrolysis. Previous *rpt* mutations have not been uniform from gene to gene and in many cases have not been mapped to the ATPase domain.

We assayed the viability of RPT substitution mutants using the plasmid shuffle method. In each case, the mutant allele was introduced on a *CEN* plasmid into a strain in which the chromosomal *RPT* gene had been deleted, and whose viability was maintained by a wild-type *RPT* gene carried on a *URA3*-marked *CEN* plasmid. Growth of colonies in the presence of 5-fluoro-orotic acid (FOA) indicates that a mutation is non-lethal, since FOA selects for the Ura$^-$ phenotype that is generated by loss of the plasmid carrying the cognate wild-type gene.

Using this assay, the *RPT* genes were resolved into three classes: the *RPT2* gene was most sensitive to mutation, since even a conservative substitution of its active-site lysine conferred lethality (Figure 2A). The second class, in which the conservative mutants were viable but the non-conservative mutants were non-viable, included *RPT3*, *RPT4* and *RPT6* (Figure 2A and B). Within this class, the relative phenotypic strengths of the mutants could be ordered on the basis of the growth

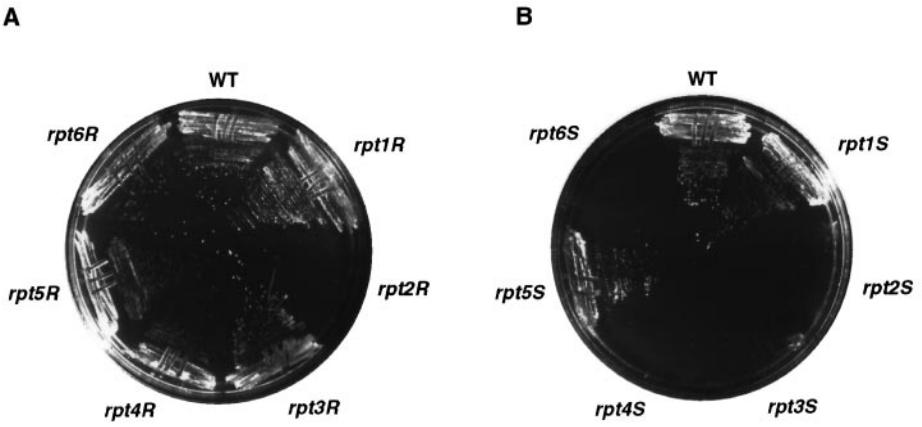

Fig. 2. Viability of strains expressing Rpt proteins with equivalent active-site lysine substitutions. (**A**) Strains expressing a given wild-type *RPT* gene from a *URA3*-marked *CEN* plasmid, as well as the same *RPT* gene with a conservative Lys to Arg (R) substitution from a *LEU2*-marked *CEN* plasmid in an appropriate *rpt* gene deletion background, were streaked onto FOA plates and allowed to grow at 30°C for 72 h. (**B**) As for (A), but with non-conservative Lys to Ser (S) substitutions.

Table I. Growth phenotypes of ATPase mutants

	Substitution	30°C	37°C	Canavanine
WT	–	+++	+++	+++
RPT1	K256R	+++	+++	+++
	K256S	++	+	–
RPT2	K229R	–	nd	nd
	K229S	–	nd	nd
	S241F	+++	+++	+++
	K229R/S241F	+	+/–	–
RPT3	K219R	+++	+++	+++
	K219S	–	nd	nd
RPT4	K297R	+	+	–
	K297S	–	nd	nd
RPT5	K228R	+	+/–	–
	K228S	+	+/–	–
RPT6	K195R	+++	++	++
	K195S	–	nd	nd

Growth of mutants compared with that of the wild type after 2 days on YPD at 30°C, YPD at 37°C or synthetic Glu media minus arginine plus 3 μg/ml canavanine. For cases marked '+/–', lethality was often observed, accompanied by some clonal outgrowth. nd, not done, owing to the lethality of the mutation under standard conditions.

phenotypes of the conservative substitutions (Table I); *rpt4R* was more strongly affected than *rpt3R* and *rpt6R*. In the third class, consisting of *RPT1* and *RPT5*, both conservative and non-conservative substitutions were tolerated, although *rpt5R* mutants grew more slowly than *rpt1R* mutants (Table I). Proteasome mutants generally display sensitivity to stress conditions such as high temperature and exposure to canavanine, an amino acid analog which interferes with protein folding and increases the flux through the ubiquitin–proteasome pathway (Heinemeyer *et al*., 1991). As expected, the mutants displayed severe phenotypes under these stress conditions (Table I). In summary, considering the properties of both conservative and non-conservative substitutions, each *RPT* gene displayed a distinct phenotype. These data imply that, to a surprising extent, the proteasomal ATPases are functionally non-redundant. In addition, the differentiation of function among the proteasomal ATPases suggests a basic distinction between the proteasome and the simple ATP-dependent proteases of prokaryotes, which contain homomeric ATPase complexes.

Isolation of an rpt2R suppressor

Of the conservative substitutions, only *rpt2R* was lethal, suggesting that Rpt2 may be of special interest. To obtain a viable *rpt2R* allele, we proceeded to identify an intragenic suppressor (see Materials and methods). A suppressor mutation in which Ser241 is replaced by Phe was chosen for analysis. Ser241 is predicted to be distant from the active site in the three-dimensional structure of the ATPase domain (Beyer, 1997). The suppressor mutation alone (*rpt2F*) had no detectable phenotypic effects in the absence of the K229R substitution (Table I; see also below), consistent with the lack of conservation of this residue among the *RPT* genes. While the suppressor mutation alleviates the lethal phenotype of the *rpt2R* mutant, the suppressor strain *rpt2RF* remains strongly growth defective, temperature sensitive and unable to grow in the presence of canavanine (Table I).

Protein turnover in the rpt mutants

In the ATP-dependent proteases of *Escherichia coli*, the ATPase domains function as specificity factors, apparently by interacting directly with substrate proteins (Gottesman *et al*., 1997). Based on this model, one explanation for both the non-redundancy among the Rpt proteins and their distinct phenotypes is that each mediates the degradation of a different set of proteins. To test this possibility, we assayed the turnover of the model substrates Lys–βgal and Ub–Pro–βgal, which are targets of the ubiquitin–proteasome pathway. Ub–Lys–βgal becomes a substrate for the N-end rule pathway following its processing by deubiquitinating enzymes to produce free ubiquitin and Lys–βgal (Varshavsky, 1992). The ubiquitin portion of Ub–Pro–βgal is not removed but rather serves as a substrate for further ubiquitination (Varshavsky, 1992). Lys–βgal and Ub–Pro–βgal are modified by different types of multiubiquitin chains (Johnson *et al*., 1995) and several proteasome mutants stabilize one substrate but not the other (Johnson *et al*., 1995; van Nocker *et al*., 1996; Fujimuro *et al*., 1998). Degradation was assayed in wild-

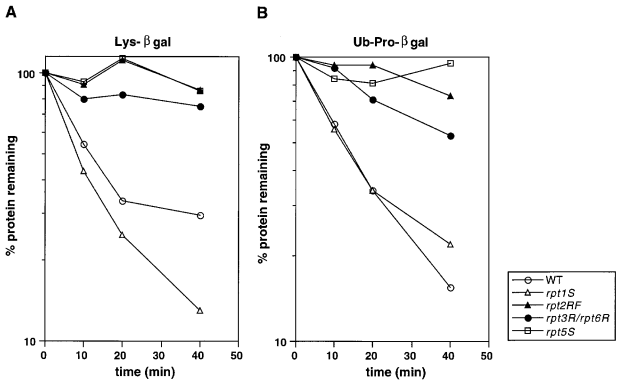

Fig. 3. Protein turnover in slow-growing rpt mutants. Plasmids expressing reporter proteins (**A**) Lys–βgal or (**B**) Ub–Pro–βgal were introduced into the various strains. Protein turnover was determined by pulse–chase analysis as previously described (van Nocker *et al*., 1996). Substrates were labeled with ^{35}S-amino acids, immunoprecipitated from cell lysates and subjected to SDS–PAGE. β-galactosidase levels were quantitated by PhosphorImager analysis and expressed as a percentage of that present during initiation of the chase. Experiments on all strains were performed three to five times and the results were averaged. The faster turnover of Lys–βgal in the *rpt1S* mutant, as compared with the wild type, is reproducible.

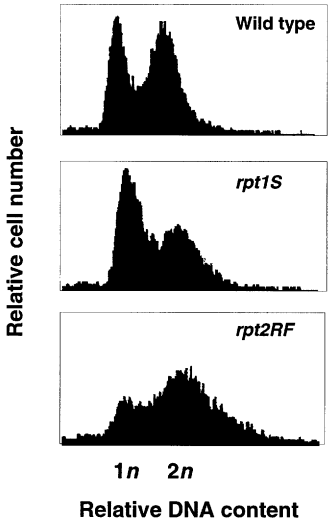

Fig. 4. FACS analysis of mutant strains. Wild-type, *rpt2RF* and *rpt1S* strains were grown at 30°C to mid-log phase, fixed with methanol, propidium iodide stained and analyzed by flow cytometry.

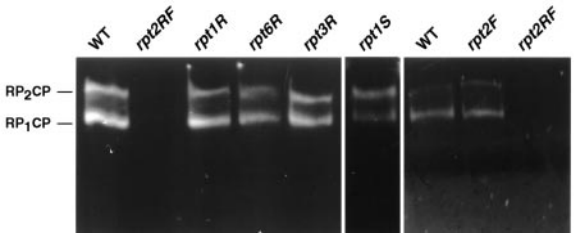

Fig. 5. Non-denaturing electrophoresis of partially purified proteasomes from wild-type and mutant strains. Lysates were fractionated using DEAE–Affigel Blue. Samples from a 50–150 mM NaCl step elution, containing ~30 μg of protein, were separated by non-denaturing PAGE. Proteasome bands were visualized *in situ* by peptidase activity against the fluorogenic substrate Suc–LLVY–AMC. The slower-migrating form represents doubly capped proteasomes (RP_2CP) and the faster form singly capped proteasomes (RP_1CP) (Glickman *et al*., 1998a).

type cells and in the more strongly growth-defective of the viable mutants: *rpt2RF*, *rpt5S* and a double mutant, *rpt3R rpt6R* (unlike the single mutants from which it is derived, *rpt3R rpt6R* is growth defective; data not shown). Strong stabilization of both substrates was observed in all three mutant strains (Figure 3), indicating that multiple ATPases are required for the degradation of a specific protein. We have obtained comparable results using heterologously expressed mammalian NF-κB as a substrate (Sears *et al*., 1998). The data suggest that these Rpt proteins generally function in a closely co-operative manner on the same substrate, rather than serve as distinct specificity factors for different substrates.

Additional pulse–chase experiments confirmed that *rpt2RF* mutants are severely affected in protein turnover as compared with other conservative mutants such as *rpt1R*, *rpt3R* and *rpt6R*. The latter mutants failed to show significant stabilization of Lys–βgal and Ub–Pro–βgal (data not shown). Thus, both cell growth and protein turnover assays showed that the *rpt2RF* phenotype is much stronger than those of the Lys to Arg substitutions in the other Rpt proteins. Additionally, the *rpt2F* mutant showed wild-type degradation of both substrates (data not shown), indicating that the *rpt2RF* protein turnover defect resulted from substitution of the invariant active-site lysine.

Interestingly, the *rpt1S* mutant, although strongly growth-defective, did not show stabilization of either Lys–βgal or Ub–Pro–βgal (Figure 3). The *rpt1S* mutant also degraded canavanyl proteins at rates comparable with the wild type (data not shown). Thus, the *rpt1S* mutant is proficient in the turnover of several proteasome substrates. Its growth defect may result from deficient turnover of a minor subset of substrates. Like many other proteasome mutants (Ghislain *et al*., 1993; Gordon *et al*., 1993; Kominami *et al*., 1997) the growth-defective *rpt* mutants generally display a pronounced G_2/M delay (Figure 4 and data not shown). This phenotype presumably reflects impaired degradation of mitosis-specific APC substrates such as B-type cyclins (Ghislain *et al*., 1993). In keeping with the exceptional nature of the *rpt1S* mutant, it showed a higher proportion of cells with a 1n DNA content, suggesting a G_1 rather than a G_2/M delay (Figure 4). Consistent with the FACS analysis, the *rpt1S* mutant also displayed a low budding index, characteristic of an early G_1 delay (Table II). Interestingly, these data indicate that the *rpt* mutants differ not only in the severity of their defects but also in the qualitative nature of the defect. These results are consistent with the suggestion that the specificity of the protein turnover effects of the *rpt1S* mutation differs significantly from those of the other *rpt* mutations.

Assembly state of mutant proteasomes

The assembly state of proteasomes in the *rpt* mutants was assayed by subjecting unpurified proteasomes to non-denaturing gel electrophoresis (Glickman *et al*., 1998a). Although ATP hydrolysis is essential for association of the regulatory and core particles *in vitro* (DeMartino *et al*., 1994; Hoffman and Rechsteiner, 1994), the ATP binding-site mutations *rpt1R*, *rpt1S*, *rpt3R* and *rpt6R* did not prevent this interaction (Figure 5). Using a fluorogenic peptide overlay assay for peptidase activity of

Table II. Budding indices of mutant strains

Strain	*n*[a]	% budded[b]	% large[c]
Wild-type	238	53	46
rpt1S	222	34	46
rpt2RF	259	64	80
rpt5S	256	69	77

[a]Total number of cells counted. Exponential cultures with comparable optical densities were taken for analysis.
[b]Percentage of total cells which were budded.
[c]Percentage of total budded cells with large buds.

the complex, two bands were observed, corresponding to proteasomes containing one (RP_1CP) or two (RP_2CP) regulatory particles (Glickman *et al*., 1998a). The mutations did not affect the relative levels of these isoforms, indicating that the affinity of the regulatory particle for the core particle was not significantly affected by these mutations. However, neither band was observed in samples from the *rpt2RF* mutant (Figure 5). This suggested either an assembly defect in the mutant or a specific effect of the mutation on peptidase activity of the complex. *rpt2RF* proteasomes were indistinguishable from wild-type in this assay (Figure 5), indicating that the defect observed for *rpt2RF* was caused by substitution of the invariant lysine.

The experiments above address the question of whether the *rpt* mutations affect the assembly of the regulatory particle and core particle to form the holoenzyme, but they do not directly address the possibility that the *rpt* mutations prevent the incorporation of mutant ATPases into the regulatory particle. For non-lethal mutations such as *rpt1S* and *rpt2RF*, incorporation of the mutant subunits into the proteasome was directly verified (see below). For lethal mutations this issue was addressed in two ways. First, each mutant allele was expressed from a high-copy-number, 2μ plasmid. If lethality were the result of a partial inhibition of subunit incorporation in the mutant, overexpression of the mutant polypeptide might be expected to drive increased incorporation and thus rescue lethality, if not fully restore the wild-type phenotype. Overexpression did not rescue the lethality of any of the mutations (data not shown). Secondly, the mutated alleles were epitope tagged and expressed in a wild-type genetic background. In those cases tested (*rpt2R, rpt3S, rpt4S* and *rpt6S*), the epitope-tagged ATPases co-purified with the proteasome (data not shown). Taken together, these experiments suggest that the effects of the ATP-binding site mutations result primarily from the inability of the mutant ATPases to function productively when properly assembled in the regulatory particle.

Peptidase defect in the rpt2RF mutant

To test whether the apparent peptidase defect and *in vivo* protein turnover defect of the *rpt2RF* mutant result from a failure to assemble proteasomes, sucrose gradient analysis was performed. In extracts from wild-type as well as *rpt1S* control cells, peptidase activity sediments predominantly in a single peak, representing a mixture of RP_1CP and RP_2CP forms of the proteasome holoenzyme (Figure 6). This peak is collected in fractions 5 and 6 of the gradient. In the *rpt2RF* extract, the peak of peptidase activity was

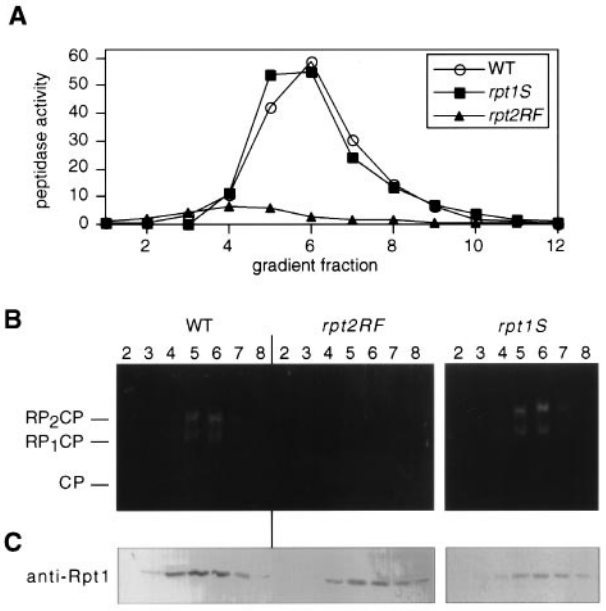

Fig. 6. Sucrose gradient analysis of wild-type and mutant proteasomes. Lysates from wild-type and mutant strains were fractionated by sucrose gradient centrifugation. (**A**) Gradient fractions were assayed for peptidase activity against Suc–LLVY–AMC. (**B**) Samples from each fraction were separated by non-denaturing PAGE and visualized by fluorogenic peptide overlay, using Suc–LLVY–AMC. (**C**) Rpt1 levels in each fraction were measured by immunoblotting with anti-Rpt1 antibody. These data show that there is no defect in Rpt1 incorporation into proteasomes in the *rpt1S* strain.

Table III. Dissociation of inactive *rpt2RF* proteasome[a] releases an active CP

	Peptidase activity	
	WT	rpt2RF
Untreated[b]	38	4
Dissociated[c]	101	100
Fold stimulation	3	25

[a]Samples are from sucrose gradient holoenzyme peak fractions (fraction 6; see Figure 6).
[b]Peptidase assay performed at 30°C as described in Materials and methods, using Suc-LLVY-AMC as a substrate. Activity values are given in arbitrary fluorescence units. The defect in *rpt2RF* activity as compared with the wild-type was essentially independent of the incubation temperature.
[c]Samples were pre-incubated in Buffer A with 500 mM NaCl and without ATP for 30 min (Glickman *et al*., 1998a). This treatment dissociates the holoenzyme and releases the core particle. The peptidase assay was performed in the presence of 0.02% SDS, which activates core particles.

reduced by approximately an order of magnitude. In addition, the small residual activity was shifted to fraction 4, corresponding to the peak of sedimentation of free core particles. These effects apparently could be accounted for by a reduction of the peptidase activity of *rpt2RF* holoenzymes.

Interestingly, when peptidase assays were carried out under conditions that dissociate the regulatory and core particles, the peptidase activity in fraction 6 from the mutant was restored to the wild-type level (Table III). These data imply that proteasome holoenzyme is present in the *rpt2RF* mutant at a level comparable with that of

wild-type, and that these proteasomes have a reduced specific activity for peptide hydrolysis. Consistent with this conclusion, immunoblotting of the sucrose gradient fractions revealed Rpt1 and Rpt6 in fractions 5 and 6 at levels approximating those of the wild type (Figure 6C and data not shown).

Based on structural studies, the channels of yeast core particles are expected to exist predominantly in a closed state (Groll *et al*., 1997). Consistent with these data, the peptidase activity of yeast core particle can be stimulated either by complex formation with the regulatory particle or by SDS treatment (Glickman *et al*., 1998a). SDS stimulates the activity of core particles ~10-fold (Glickman *et al*., 1998a). Interestingly, the 25-fold stimulation of peptidase activity of the *rpt2RF* proteasomes by SDS (Table III) exceeded that of the core particle itself. Thus, whereas wild-type regulatory particles stimulate the core particle, the mutant regulatory particle has an inhibitory influence. The extent of inhibition is likely to be underestimated in this experiment due to the presence of free core particles in the *rpt2RF* proteasome holoenzyme peak fraction (data not shown). In summary, these data suggest that, among the proteasomal ATPases, Rpt2 has a specialized function involving either the peptidase activity of the complex or access of peptides to the lumen of the core particle.

Purification of mutant rpt2RF proteasomes

To verify the *rpt2RF* peptidase defect, proteasomes were purified from *rpt2RF* mutants. Given the proteolytic inactivity of the *rpt2RF* proteasome, purification could be followed only by immunoblotting against subunits of the particle. By Coomassie Blue staining and immunoblotting (Figure 7A–C), the subunit profile of the purified *rpt2RF* proteasome was indistinguishable from that of the wild type, suggesting that the *in vitro* defect is directly due to the point mutation. In particular, the mutant Rpt2 subunit was present, as indicated in Figure 7B and confirmed by mass spectrometry (data not shown).

Purified *rpt2RF* proteasomes were electrophoresed on a non-denaturing gel, which was then subjected to the fluorogenic peptide overlay assay. The purified complex had strongly reduced peptidase activity (Figure 7D). To confirm that intact proteasomes were indeed present in the gel, it was stained with Coomassie Blue (Figure 7E). An intense band was observed to co-migrate with the RP_2CP form of the wild-type enzyme. Taken together, Figure 7D and E demonstrate a strong defect in the peptidase activity of *rpt2RF* proteasomes. Moreover, they reveal an additional biochemical phenotype of the mutant: *rpt2RF* proteasomes were found only in the RP_2CP isoform. Since the RP_1CP and RP_2CP isoforms have comparable peptidase activity in the wild-type enzyme (Figure 7D and E), this assembly phenotype cannot explain the peptidase defect.

The excess of RP_2CP over RP_1CP could result from an increase in the abundance of RP complexes *in vivo* as a consequence of the *rpt2RF* mutation. Perhaps more likely, the effect may be based on differential stability of the two complexes during the course of purification. If so, the excess of RP_2CP over RP_1CP in the mutant would suggest that the binding of RP complexes to the two ends of the CP cylinder is not independent. A similar conclusion

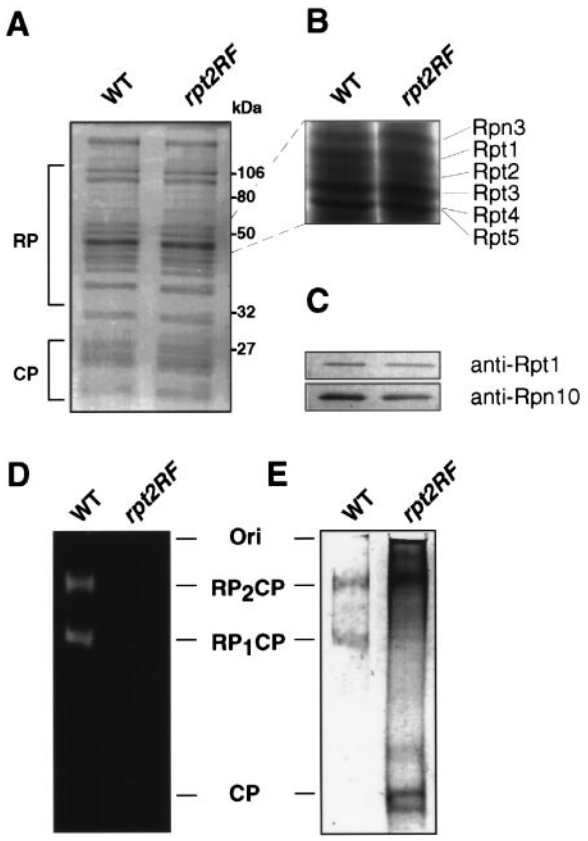

Fig. 7. Purification of *rpt2RF* proteasomes. Samples from the final purification step of the wild-type and *rpt2RF* proteasomes were analyzed on by SDS–PAGE. (**A** and **C**) 12% polyacrylamide gel. (**B**) 10–20% polyacryamide gradient gel. Protein bands were (A and B) stained with Coomassie Blue and (C) immunoblotted with the indicated antibodies. (**D**) Proteasome bands from the final purification steps were visualized in a non-denaturing gel by fluorogenic (Suc–LLVY–AMC) peptide overlay. (**E**) Proteasome bands shown in (D) were stained with Coomassie Blue. Throughout purification, the presence of inactive mutant proteasome was tracked by immunoblotting and Coomassie Blue staining. In (A) the highest molecular mass band must be either a contaminant or a loosely associated subunit (data not shown). Band assignments in panel B are based on the data of Glickman *et al.* (1998a). The identity of the Rpt2RF protein was further confirmed by trypsin digestion, followed by mass spectrometry.

was recently reached in studies of the *in vitro* association of purified CP and RP/PA700 from mammals (Adams *et al*., 1997). However, in these experiments, the apparent co-operativity of assembly was weak. Interesttingly, the *rpt2RF* mutation appears to strongly enhance the stability of the RP_2CP form in comparison with RP_1CP.

As previously observed, wild-type proteasomes stringently require ATP for the breakdown of ubiquitin–protein conjugates (Figure 8). However, *rpt2RF* proteasomes failed to degrade ubiquitin–protein conjugates in both the presence and absence of ATP (Figure 8). This defect was not a result of instability of the purified *rpt2RF* proteasomes, since, over the time course of the assay, *rpt2RF* proteasomes remained assembled in the RP_2CP form, as assayed by non-denaturing gels (data not shown). This experiment confirms the critical role of Rpt2 in proteasome function and shows that the *in vivo* protein turnover defects of the *rpt2RF* mutant reflect intrinsic properties of the mutant proteasomes.

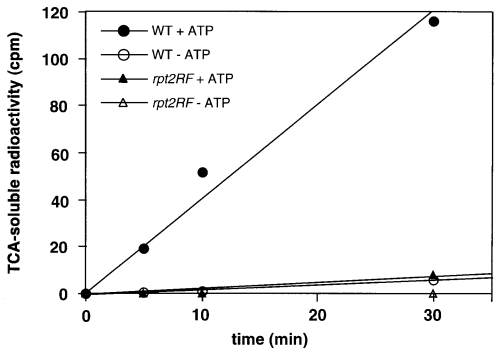

Fig. 8. Breakdown of ubiquitinated lysozyme by the wild-type and rpt2RF proteasomes. Proteasomes purified from wild-type and *rpt2RF* strains were tested for their ability to degrade ubiquitinated ^{125}I-labeled lysozyme in the presence (filled symbols) or absence (hollow symbols) of ATP. Degradation is measured as the release of ^{125}I-CPM into a TCA-soluble form.

Discussion

Multiple roles for ATP in the proteasome

That intracellular proteolysis involves a complex proteolytic mechanism was initially suggested by its ATP dependence (Simpson, 1953). Subsequent work by Hershko, Ciechanover and their colleagues showed that proteins can be targeted for degradation by conjugation to ubiquitin in an ATP-dependent reaction (Hershko and Ciechanover, 1992). With the discovery of the proteasome as the ubiquitin-dependent protease (Hough *et al*., 1987), a second ATP-dependent step was recognized in this pathway. The identification of six ATPases in the proteasome (Dubiel *et al*., 1992, 1993; Rubin *et al*., 1996; Glickman *et al*., 1998a), indicated that ATP could have multiple roles which would be difficult to resolve by studying only the wild-type enzyme. Using yeast as a model system, site-directed ATPase mutants allow the problem to be studied at the level of individual active sites, and to be approached through a combination of *in vivo* and *in vitro* methods.

Since the proteasomal ATPases are strongly related to one another in sequence, we expected to find extensive functional redundancy among them. Instead, major phenotypic effects were observed for substitutions in all six of the proteasomal ATPases. The strength of this requirement varied from subunit to subunit such that a hierarchy of phenotypic strengths was observed. The extreme cases were *RPT2*, for which even a conservative substitution of the invariant lysine was lethal, and *RPT1*, for which a non-conservative substitution engendered no general defect in degradation. The non-conservative substitution in *RPT5* was also non-lethal, but unlike *rpt1S*, this mutation resulted in a strong, apparently general degradation defect (Figure 3). Non-conservative lysine substitutions in the three remaining genes were lethal. Within this group, differences were observed in the phenotypic strengths of the conservative substitution mutants (Table I). The non-redundancy of the proteasomal ATPase activities is particularly surprising given their multiplicity, and stands in contrast to the three peptidase activities of the proteasomal core particle, which are predominantly redundant *in vivo* (Arendt and Hochstrasser, 1997; Heinemeyer *et al*., 1997)

Rpt2 linked to peptide hydrolysis

More striking than the differences in phenotypic strength were the qualitative differences in the phenotypes observed. In particular, one mutant (*rpt2RF*) showed an unexpected defect in peptidase activity. Purified proteasomes from this mutant also differed from those of the wild-type in that they existed almost exclusively in the RP_2CP isoform. A strong indicator of the *in vivo* state of the complex is sucrose gradient analysis, because it is a single-step procedure and avoids exposure to high salt concentrations, which may destabilize the complex. In these experiments, *rpt2RF* proteasomes proved to be properly assembled (Table III), despite their lower peptidase activity.

The defect of *rpt2RF* proteasomes in hydrolyzing small peptides that have no secondary structure indicates that specific proteasomal ATPases have functions other than, or in addition to, unfolding of protein substrates. This is the first observation to link a proteasomal ATPase to peptide hydrolysis. The regulatory particle was previously known to stimulate the peptidase activity of the core particle (Chu-Ping *et al*., 1992; Glickman *et al*., 1998a), but the role of the ATPases in this stimulation has been unclear. Moreover, the *rpt2RF* effect is novel because it uncouples the stimulation of peptidase activity from the role of ATP in holoenzyme assembly. The stimulation of peptidase activity that results from association of the regulatory particle with the core particle has generally been attributed to an allosteric effect on the proteolytic active sites. An alternative interpretation, based on access of the substrate to the lumen of the core particle, is suggested by the closed state of the channel in the crystal structure of the yeast core particle (Groll *et al*., 1997). Interestingly, the *rpt2RF* data suggest that the channel can assume open and closed states within an intact holoenzyme.

Whether the *rpt2RF* mutation results in a defect in allosteric control of the peptidases or a defect in channel gating remains to be rigorously determined. However, we favor the channel gating model for three reasons: first, the channel gating hypothesis could be eliminated if the peptidase defect of *rpt2RF* proteasomes were restricted to one of the three active sites of the core particle. This is not the case: peptides known to be hydrolyzed at each of the active sites (Arendt and Hochstrasser, 1997; Gerlinger *et al*., 1997) are degraded more slowly by the mutant complex (data not shown). These data also imply that if an allosteric change is propagated to the proteolytically active β subunits, it must affect the particle as a whole. Secondly, since the regulatory particle is in direct contact with the channel-forming α subunits, but not the proteolytically active β subunits (Peters *et al*., 1993), it is simpler to postulate a channel gating effect; such an effect would not have to be propagated through an interposed ring complex. Thirdly, the structural data provide significant support for the existence of a gating mechanism, whereas there is as yet little evidence for allosteric control of the peptidase sites by the regulatory particle.

Assuming that the *rpt2RF* mutation causes a channel gating defect, the lack of such an effect in four other *rpt* mutants (Figure 5) suggests that the function of modulating the state of the proteasome channel is a specialized function of Rpt2. However, it should be noted that further

characterization of the lethal *rpt* mutants will be required to test whether this role of Rpt2 is unique. It is also uncertain whether the possible channel gating defect underlies the *in vivo* defects in the turnover of ubiquitin–protein conjugates.

Unique phenotype of the Rpt1S mutant

Like Rpt2, Rpt1 appears to play a qualitatively distinct role in the proteasome. Although the *rpt1S* phenotype was not as strong as that of the other non-conservative substitutions, the *rpt1S* mutants were slow-growing, temperature-sensitive and inviable in the presence of canavanine. Nonetheless, *rpt1S* mutants failed to exhibit a general defect in protein turnover. The lack of correlation between effects on growth rate and protein turnover departs from the pattern observed for the other *rpt* mutants. The distinctness of the *rpt1S* phenotype was best reflected in the cell cycle profile of the mutant; in contrast to other *rpt* mutants, which have a high proportion of cells in G_2/M phase, *rpt1S* mutants accumulated in G_1 with a low budding index. This and other phenotypic effects of the *rpt1S* mutation could reflect protein stabilization effects that are restricted to specific substrates. It is interesting that another *rpt1* mutation (*cim5-1*) has been isolated which does stabilize Lys–βgal and Ub–Pro–βgal, unlike *rpt1S* (Ghislain *et al*., 1993). The site of the *cim5-1* mutation is unknown. Taken together, these data suggest that, for some substrates, the participation of Rpt1 in their turnover is not critically dependent on an intact ATP-binding site.

There are strong differences between the phenotypes of *rpt1* and *rpt2* mutants, and yet the mammalian homologs of these proteins have been proposed to associate specifically with each other (Richmond *et al*., 1997). Our data have no bearing on whether such pairwise associations occur within the proteasome, but they do indicate that, if such a pairing exists, it is unlikely to underlie a closely parallel function for the two proteins. For example, the *rpt1S* mutant does not have a deficiency of peptidase activity as seen in *rpt2RF* mutants (Figure 5), and the two mutations have different effects on cell cycle progression (Figure 4).

Co-ordination of ATP hydrolysis in multisubunit ATPase complexes

In both prokaryotes and eukaryotes, oligomeric ATPase ring complexes play central roles in protein folding and degradation. The best understood of these ATPase complexes is GroEL (Fenton and Horwich, 1997), which is required for folding of many nascent proteins in *E.coli*. GroEL is a homo-oligomer composed of two rings, each containing seven subunits. While ATP hydrolysis by subunits in a given ring is co-ordinated through positive co-operativity, the activity of the two rings is co-ordinated by inter-ring negative co-operativity. Thus, ATP hydrolysis by GroEL subunits is a highly co-ordinated process. Closely related to GroEL is a eukaryotic complex, the Cct chaperonin. This is a hetero-oligomer containing eight distinct, putative ATPase subunits, the only particle known to have a greater diversity of ATPases than the proteasome. In contrast to our results with the proteasome, extensive mutagenesis has thus far failed to demonstrate a function that is essential *in vivo* for any of the individual ATP-binding motifs in the complex (Lin and Sherman, 1997; Lin *et al*., 1997). One explanation for these results is that, in a strongly co-operative system, the conformational transitions driven by the ATPase cycle may be transmitted from wild-type to mutant subunits (Lindsley and Wang, 1993; Lin and Sherman, 1997). Such a mechanism has been demonstrated most elegantly for topoisomerase II (Lindsley and Wang, 1993; Berger *et al*., 1996).

Given this background, it is surprising that the majority of ATP-binding sites in the proteasome are required for function. Although we have not yet measured the effect of the A motif substitutions on ATP binding and hydrolysis by purified proteasomes, the mutations clearly have dramatic effects, and the data place limitations not only on the functional redundancy of the Rpt subunits, but perhaps also on the extent of allosteric communication among them. Our results indicate that multiple ATPases function jointly in the degradation of a given protein, but they do not imply positive co-operativity among the proteasomal ATPases. Indeed, given that closely coupled ATPases may drive conformational cycles in a mutant partner (Berger *et al*., 1996), the strong *rpt* phenotypes that we have observed do not favor this model. Moreover, kinetic studies on the mammalian proteasome have so far failed to detect a co-operative effect of ATP concentration on the ATP hydrolysis rate (DeMartino *et al*., 1994; Hoffman and Rechsteiner, 1996).

The mutants described here will provide a valuable resource for future mechanistic studies of the proteasome. For example, purified mutant proteasomes can be used to test proposed roles of specific Rpt proteins in gating of the proteasome channel and unfolding of proteolytic substrates. Unfolding of proteolytic substrates, and their translocation through the interior cavities and channels of the proteasome, are likely to be guided by ATP-driven conformational changes in the particle. A comprehensive view of this process may ultimately emerge through gaining an understanding of the mechanistic role of each ATPase individually.

Materials and methods

Yeast strains, media and genetic techniques

Strain SUB62 (*MAT***a** *his3-Δ200 lys2-801 leu2-3,112 trp1-1 ura3-52*) was used as a wild-type control (Finley *et al*., 1987), and all phenotypic analyses were carried out with *MAT***a** derivatives. Yeast cultures were grown at 30°C unless otherwise noted. YPD media consisted of 1% yeast extract, 2% Bacto Peptone and 2% glucose. YPR media consisted of 1% yeast extract, 2% Bacto Peptone and 2% raffinose. Synthetic media consisted of 0.7% Difco yeast nitrogen base supplemented with amino acids, uracil and adenine, as described previously (Rose *et al*., 1990) and either 2% glucose (synthetic Glu media), 2% galactose (synthetic Gal media), 2% raffinose and 2% galactose (synthetic RafGal media). Uracil was omitted where necessary for plasmid selections, methionine was omitted for pulse-labeling experiments and arginine was omitted from medium supplemented with canavanine. Standard techniques were used for Li–acetate yeast transformations and tetrad analysis (Gietz and Sugino, 1988; Rose *et al*., 1990). Flow cytometry was performed at the Dana Farber Cancer Institute Flow Cytometry Facility after cells had been grown to log phase, sonicated, fixed, treated with RNase and pepsin, and stained with propidium iodide (Hutter and Eipel, 1979). To determine the budding indices log phase cells were applied to a hemocytometer and the number of non-budded, large-budded and small-budded cells were counted.

Construction of wild-type and mutant RPT yeast expression plasmids

Standard PCR protocols were used to amplify the *RPT1–RPT6* genes from yeast genomic DNA (strain SUB62), and the fragments were

Table IV. Yeast strains and plasmids used in this work

	FOA-reverted[a]		Prior to FOA-reversion[b]	
	Strain	Plasmid(s)	Strain	Plasmid(s)
RPT1	DY85	Dp73	DY75	Dp5, Dp73
RPT2	DY188	Dp100	DY158	Dp4, Dp100
RPT3	DY92	Dp74	DY79	Dp9, Dp74
RPT4	DY218	pUB328	DY215	pUB309, pUB328
RPT5	DY61	Dp41	DY48	Dp12, Dp41
RPT6	DY104	Dp80	DY102	Dp14, Dp80
rpt1R	DY98	Dp87	DY95	Dp5, Dp87
rpt2R	–	–	DY159	Dp4, Dp101
rpt3R	DY93	Dp75	DY80	Dp9, Dp75
rpt4R	DY219	pUB326	DY217	pUB309, pUB326
rpt5R	DY155	Sp12	SY152	Dp12, Sp12
rpt6R	DY100	Dp89	DY97	Dp14, Dp89
rpt1S	DY106	Dp91	DY103	Dp5, Dp91
rpt2S	–	–	DY222	Dp4, Dp119
rpt3S	–	–	DY81	Dp9, Dp76
rpt4S	–	–	DY216	pUB309, pUB327
rpt5S	DY65	Dp43	DY52	Dp12, Dp43
rpt6S	–	–	DY96	Dp14, Dp88
rpt2F	DY57	Dp37	SY122	Dp4, Dp37
rpt2RF	DY62	Dp42	DY44	Dp4, Dp42
rpt3R/rpt6R[c]	SY147	Dp75, Sp5	DY49	Dp14, Dp75, Sp5

[a]Strain containing *LEU2*-marked *CEN* plasmid expressing a wild-type or mutant *RPT* gene over the corresponding chromosomal deletion.
[b]Strain containing *LEU2*-marked *CEN* plasmid expressing a wild-type or mutant *RPT* gene and a *URA3*-marked *CEN* plasmid expressing a wild-type *RPT* gene over the corresponding chromosomal deletion.
[c]Mutant containing chromosomal deletions of *RPT3* and *RPT6* covered by plasmids expressing corresponding genes with Lys to Arg substitutions. The plasmid Sp5 is marked with *TRP1*.

cloned into pUC19. The clones were sequenced on both strands and found to be free of mutations. One *RPT2* clone was recovered with a serendipitous Taq polymerase-introduced mutation (S241F). Standard methods were used for DNA manipulation and sequencing (Sambrook *et al*., 1989). Sequences of oligonucleotides used in this work are available on request.

The Lys codon of the A motif in each *RPT* gene was mutated using PCR methods (Figure 1) (Higuchi *et al*., 1988). The mutated DNA fragments were cloned into the pUC19-borne ATPase genes, and the resulting clones sequenced. The Lys to Arg mutation was also introduced into *rpt2–S241F*.

A set of plasmids designed to express wild-type and mutant versions of the Rpts from a single (*RPT1*-derived) promoter was constructed. Northern blot analysis revealed that *RPT1*, *RPT2*, *RPT3*, *RPT5* and *RPT6* express RNA to closely comparable levels at both 30 and 37°C (data not shown), suggesting that the *RPT* promoters are all functionally equivalent. Using a PCR protocol (Higuchi *et al*., 1988), a DNA fragment containing the *RPT1* 5′ UTR, a multiple cloning site (MCS) and the *RPT1* 3′ UTR was constructed. The fragment was subcloned as a *Hin*dIII–*Kpn*I fragment into YCplac33 and YCplac111 (Gietz and Sugino, 1988), producing Dp2 and Dp22, respectively. The wild-type versions of all six *RPT* genes were subcloned into the MCS of Dp2 and Dp22, and the mutant versions into Dp22 (Table IV).

Construction of RPT deletion strains expressing wild-type or mutant RPT genes

Haploid cells, containing a given *RPT* gene deletion covered by a *URA3*-marked *CEN* plasmid (Dp2) expressing the wild-type gene from a *RPT1* promoter, were described previously (Glickman *et al*., 1998a). *LEU2*-marked *CEN* plasmids, carrying wild-type or mutant versions of a given *RPT* gene, were introduced into the appropriate strain (Table IV). Upon FOA-reversion (Rose *et al*., 1990) strains were obtained with only the *LEU2*-marked plasmids (Table IV). Wild-type *RPT* genes expressed from these plasmids complemented the corresponding chromosomal deletion mutants. Ub–Pro–βgal and Lys–βgal were expressed from *URA3*-marked plasmids (Bachmair *et al*., 1986).

Sucrose density centrifugation, affinity chromatography and purification of the proteasome

Buffer A, consisting of 50 mM Tris pH 7.4, 5 mM $MgCl_2$, 10% glycerol, 1 mM adenosine 5′ triphosphate (Grade 1, Sigma), and 1 mM dithiothreitol, was used in all *in vitro* experiments unless otherwise stated. Clarified yeast extract was fractionated by sucrose gradient centrifugation as described (Peters *et al*., 1993). Purification of the proteasome by conventional anion exchange and gel filtration chromatography has been described previously (Rubin *et al*., 1996; Glickman *et al*., 1998a). Protein determinations were carried out using the method of Bradford, using bovine serum albumin as the standard (Bradford, 1976).

Immunological techniques

For immunoblotting experiments, protein samples were resolved on SDS–PAGE and transferred onto nitrocellulose membranes as described previously (Rubin *et al*., 1996; Glickman *et al*., 1998a). Primary antibodies were visualized with alkaline phosphatase-labeled goat anti-rabbit immunoglobulins or by binding to ^{125}I-protein A. Quantitation was achieved using a PhosphorImager and ImageQuant software package (Molecular Dynamics).

Pulse–chase assays

Methods for pulse–chase and immunoprecipitation were as described peviously (van Nocker *et al*., 1996). Briefly, cells were grown to exponential phase at 30°C in synthetic medium supplemented with 2% raffinose, 2% galactose and amino acids. Immunoprecipitated samples were separated by SDS–PAGE, and the dried gels exposed to PhosphorImager cassettes. Band intensities of βgal were determined for at least three pulse–chases for each strain, using the ImageQuant software package, and were averaged and graphed using KaliedaGraph (Abelbeck Software).

Assays of proteasome activity

Protein samples were assayed for their ability to degrade Suc–LLVY–AMC, Boc–LRR–AMC and z–LLE–BNA (Bachem) in solution in the presence of 1 mM ATP or in the presence of 1 mM ATP and 0.02% SDS, as described previously (Rubin *et al*., 1996). Protein samples were resolved by non-denaturing polyacrylamide gel electrophoresis (PAGE) and treated with an overlay of Suc–LLVY–AMC and the proteasome was visualized upon exposure to UV light as described (Glickman *et al*., 1998a). Ub-conjugated lysozyme breakdown assays were performed essentially as described previously (Rock *et al*., 1994).

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