ATP-binding by proteasomal ATPases regulates cellular assembly and substrate-induced functions of the 26S proteasome

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Background: ATPase subunits mediate 26S proteasome assembly and function.

Results: Defective ATP binding by some ATPase subunits inhibits proteasome assembly, and proteasomes harboring an ATP-binding-defective subunit are impaired for proteolysis and for substrate-stimulated gating and ATPase activity.

Conclusion: Proteasome requires normal ATP binding for assembly and function.

Significance: Protein substrates promote their own degradation by inducing ATP-dependent proteasome functions.

SUMMARY

We examined the role of ATP-binding by six different ATPase subunits (Rpt1-6) on the cellular assembly and molecular functions of mammalian 26S proteasome. Four Rpt subunits (Rpt1-4) with ATP binding mutations were incompetent for cellular assembly into 26S proteasome. In contrast, analogous mutants of Rpt5 and Rpt6 were incorporated normally into 26S proteasomes in both intact cells and an in vitro assembly assay. Surprisingly, purified 26S proteasomes containing either mutant Rpt5 or Rpt6 had normal basal ATPase activity and substrate gate opening for hydrolysis of short peptides. However, these mutant 26S proteasomes were severely defective for ATP-dependent in vitro degradation of ubiquitylated and non-ubiquitylated proteins and did not display substrate-stimulated ATPase and peptidase activities characteristic of normal proteasomes. These results reveal differential roles of ATP binding by various Rpt subunits in proteasome assembly and function. They also indicate that substrate-stimulated ATPase activity and gating depend on the concerted action of a full complement of Rpt subunits competent for ATP binding and that this regulation is essential for normal proteolysis. Thus, protein substrates appear to promote their own degradation by stimulating proteasome functions involved in proteolysis.

Intracellular protein degradation requires metabolic energy. Although the cleavage of peptide bonds per se is exergonic, protease machines found in all domains of life use ATP binding and hydrolysis to regulate their function (1;2). In eukaryotes, most ATP-dependent intracellular protein degradation is catalyzed by the 26S proteasome, a 2.5 MDa complex that degrades polyubiquitylated proteins and certain non-ubiquitylated proteins(3;4). ATP is likely used to promote structural features of the proteasome required for proteolysis and to support and integrate multiple events of substrate processing prior to peptide bond cleavage. However, a comprehensive understanding of how ATP fuels the 26S proteasome for proteolysis is lacking.

The 26S proteasome is composed of a cylinder-shaped 20S proteolytic complex bound at one or both ends to a PA700 (19S) ATPase regulatory complex (5). The interface of these complexes consists of axially-abutting rings of 20S and PA700 subunits (6;7). The center of the heteroheptameric 20S ring forms a narrow pore for entry of substrates to the interior of the four-ring 20S cylinder where protease active sites reside (8;9). The pore is reversibly gated by flexible
N-termini of the 20S subunits (10). The hexameric PA700 ring is composed of homologous ATPases (termed Rpt1-6) of the AAA protein family (11). AAA proteins feature conserved Walker A and Walker B domains that bind and hydrolyze ATP, respectively (12;13). C-terminal residues of several Rpt subunits induce an open gate conformation upon binding to pockets between 20S α subunits (14-16). Gate opening can be induced directly by binding of isolated C-terminal peptides of gating-competent Rpt subunits in the absence of ATP (5;17), but requires ATP binding in intact proteasomes (18-20). Yeast 26S proteasomes containing an ATP-binding defective mutant Rpt2 subunit display defective gating (21). Analogous mutants of other Rpt subunits have disparate effects on gating and on other features of proteasome function, supporting the general conclusion that the six homologous Rpt proteins have largely non-redundant roles (22).

Rpt subunits have other critical structural and functional features required for normal 26S proteasome action. For example, the Rpt interacts with several PA700 subunits oriented proximally to the 20S complex, and forms an interface with another set of PA700 subunits oriented distally to the 20S proteasome (6;23;24). These various non-ATPase PA700 subunits provide the 26S proteasome with polyubiquitin chain-binding sites (25-28), deubiquitylating activities (29-32), and docking sites for reversibly-associated regulatory proteins (26;33;34). Thus, the Rpt subunit ring physically bridges the 20S proteasome and elements of PA700 that prepare substrates for degradation. This topological feature enables the Rpt ring to use its ATP-binding and hydrolyzing functions to coordinate and integrate multiple and diverse processes required for proteasomal proteolysis. For example, ATP binding and hydrolysis regulate polyubiquitin chain binding and subsequent substrate engagement (35). Subsequent rounds of ATP binding and hydrolysis probably promote alternating conformations of the Rpt ring that transmit mechanical force to substrates for substrate unfolding and translocation to the 20S proteasome (36;37). Substrate processing also requires disassembly of the polyubiquitin chain, which cannot traverse the narrow 20S substrate entry pore. As with peptide bond hydrolysis, deubiquitylation per se is not ATP-dependent, but becomes ATP-dependent when integrated with overall substrate degradation (30;32). This feature may reflect an enforced mechanistic linkage of deubiquitylation to other steps in substrate processing to prevent loss of binding affinity prior to committed degradation (35).

In addition to regulating the function of intact 26S proteasome, Rpt subunits play important roles in 26S proteasome assembly. Interactions between C-terminal residues of Rpt subunits and 20S proteasome are critical determinants of 26S proteasome formation (38-41). Certain Rpt subunits lacking C-terminal residues cannot assemble into 26S proteasome in intact cells. Moreover, removal of C-terminal residues from any of several Rpt subunits in purified PA700 prevents its assembly into 26S proteasome in an in vitro assembly reaction with 20S proteasome (5). 26S proteasome assembly in this reaction depends on ATP binding, but not hydrolysis (5). This effect may reflect ATP-induced conformational changes in Rpt subunits that optimize the availability of C-terminal residues for 20S proteasome binding. Such a mechanism appears to be conserved among other Rpt-like ATPases and their interactions with proteasomes. For example, PAN, an archeabacterial AAA protein ortholog of eukaryotic Rpt subunits, also requires ATP binding to associate with and promote gating of 20S proteasome in vitro (18). Nevertheless, several aspects of the physiologic significance of ATP-dependent proteasome assembly remain unclear. First, the exact cellular pathway of 26S proteasome assembly remains undefined (42;42). Despite some supporting evidence, it is not unambiguously established that 26S proteasome is assembled in cells by binding of intact PA700 to 20S proteasome. Second, conflicting results regarding the role of ATP binding by Rpt subunits on cellular assembly of 26S proteasome have been reported. For example, ATP-binding mutations in individual yeast Rpt subunits produced a range of effects that vary according to the subunit (22). As with other features of proteasome function (43), these findings suggest non-redundant roles for Rpt subunits in ATP-dependent proteasome assembly. In contrast, a recent report indicated that ATP binding mutations of any Rpt subunit blocked 26S proteasome assembly in mammalian cells (44).

This work had two related initial goals: first, to determine the general and individual roles of ATP binding by Rpt subunits in 26S proteasome as-
assembly in intact mammalian cells and second, to evaluate the physiologic significance of ATP-dependent in vitro assembly of 26S proteasome from purified 20S proteasome and PA700. Our results show that defective ATP binding differentially affected cellular proteasomal assembly of Rpt subunits and unexpectedly allowed us to study of role of ATP binding on functions purified 26S proteasomes harboring either of two different single ATP binding-defective Rpt subunits. Results of these studies reveal critical roles of ATP binding in multiple aspects of proteasome function and provide insights to molecular mechanisms of ATP-regulated proteasome activity.

EXPERIMENTAL PROCEDURES

**DNA constructs.** cDNAs encoding each full-length wild-type human Rpt subunit (Rpt1-6; PSMC2, PSMC1, PSMC4, PSMC6, PSMC3, and PSMC5, respectively) were prepared as described previously (39). For each cDNA, a conserved lysine residue in the Walker A motif (GPPGTGTK) known to be essential for ATP binding was mutated to alanine (45;46). The mutant cDNA was subcloned into pIRESpuro3 expression vector (Clontech) featuring N-terminal FLAG epitope (Supplemental Figure 1). Each construct was confirmed by DNA sequencing. As reported previously, wild-type Rpt2 was modified, apparently by proteolysis, upon stable expression in HEK293 cells (39). In contrast Rpt2 lacking the last three (C-terminal) residues was expressed without modification and was assembled normally into 26S proteasome. Therefore, the C-terminally truncated construct was used as the “wild-type” control for Rpt2 bearing an ATP-binding mutation.

**Preparation of HEK293 cell lines with stable expression of FLAG-tagged Rpt subunits containing a Walker A ATP-binding mutant.** HEK293 cells stably expressing wild-type or ATP-binding mutant FLAG-Rpt subunits were established and cultured as described previously (39). Cells expressing these proteins were morphologically indistinguishable from and grew at similar rates to normal HEK293 cells.

**Preparation of cell extracts.** HEK293 cells lines were grown to approximately 90% confluence, harvested and washed with phosphate-buffered saline. Cells were disrupted in ice-cold buffer consisting of 50 mM Tris-HCl, pH 7.5 at 4°C, 0.05% NP-40, 1 mM ATP, 5 mM MgCl2, and 1 mM β-mercaptoethanol by 15 passages through a 27-gauge needle. The lysates were centrifuged to remove debris to obtain a crude soluble fraction. Expression of Rpt proteins was determined by western blot analysis using anti-FLAG M2 antibody (Sigma, St. Louis, MO) and corresponding anti-Rpt antibodies.

**Glycerol Density Gradient Centrifugation.** Glycerol density gradient centrifugation was conducted as described previously using 12.5-40% linear glycerol gradients (Koulich et al, 2007). Gradient buffer consisted of 20 mM Tris-HCl, pH 7.5 at 4°C, 1 mM β-mercaptoethanol, 5 mM MgCl2, and 1 mM ATP.

**Affinity purification of FLAG-Rpt protein complexes.** FLAG-Rpt protein complexes were affinity purified on anti-FLAG-beads as described previously (39).

**Purification of proteasome complexes from bovine red blood cells.** 26S proteasome, latent 20S proteasome, and PA700 were purified from bovine red blood cells as described previously (20;47;48).

**Assays of proteasome activity.** Proteasome activity was measured by the hydrolysis of peptide substrates including Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC) as described previously (20). Proteasome degradation of protein substrates was measured by the degradation of [methyl-14C]-casein and polyubiquitylated-Sic, as described previously (47;49).

**Assay of 20S proteasome activation by PA700.** Proteasome activating activity by PA700 binding was assayed as described previously (20). In brief, purified PA700 was incubated with 1 nM purified latent 20S proteasome from bovine red blood cells. After incubation for 30 mins in 45 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 200 μM ATP, 5 mM MgCl2, in a final volume of 50 μl, proteasome activity was assayed by addition of 200 μl of Suc-Leu-Leu-Val-Tyr-AMC to a final
concentration of 100 μM. Control assays included exclusion of either PA700, 20S proteasome, or both.

**Preparation of polyubiquitylated Sic and unanchored polyubiquitin chains.** Polyubiquitylated 6His-Sic was prepared as described previously (49). In brief, 6His-Sic containing a PY (Pro-Tyr) motif fused to the C-terminus, was expressed in *E. coli* and purified by affinity chromatography on nickel beads. The purified 6His-Sic was incubated with purified recombinant E1, E2 (Ubc4), E3 (Rsp5), and ubiquitin. A high molecular weight fraction of polyubiquitylated Sic (Mr > 150,000) was isolated by glycerol density gradient centrifugation and dialyzed extensively against a buffer consisting of 20 mM Tris-HCl, pH 7.6 and 100 mM NaCl. Unanchored polyubiquitin chains were prepared by incubation of E1, GST-E2<sub>25K</sub> and ubiquitin. Unanchored chains were separated from GST-E2<sub>25K</sub> by chromatography on glutathione beads. A high-molecular weight fraction of polyubiquitin chains (Mr > 100,000) was prepared by glycerol density gradient centrifugation.

**Assay of ATPase activity.** ATPase activity was determined by measuring the rate of hydrolysis of [γ<sup>32</sup>P] from [γ<sup>32</sup>P]-ATP (50). Assays contained 45 mM Tris-HCl, pH 7.8, 11 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 0.2 mM ATP, and proteins specified in individual experiments in a final volume of 25 μl. After incubation at 37°C, free γ<sup>32</sup>P<sub>O</sub><sub>4</sub> was quantified. All assays were conducted under conditions in which the rate of phosphate hydrolysis was linear with respect to time and enzyme concentration. To compare the specific ATPase activities directly among various FLAG-protein complexes, we isolated affinity-purified samples by glycerol density gradient centrifugation. Gradient fractions were analyzed by western blotting for the distribution pattern of FLAG protein, and for other selected proteasome subunits such as Rpt2, Rpt 5, and Rpn12. Gradient fractions also were assayed for ATPase activity and peptide hydrolyzing activity using the substrate Suc-Leu-Leu-Val-Tyr-AMC. The combined protein and activity distribution patterns permitted identification of gradient fractions containing 26S proteasome and/or PA700 based on the known sedimentation positions of these complexes. This procedure separated 26S proteasome and PA700 from one another and from any FLAG-containing complexes not incorporated into either of them. Proteasome complexes containing equal content of representative subunits, based on western blotting and independent protein determination were assayed for ATPase activity, peptidase activity, and protease activity with protein substrates. Parallel analysis and comparison was conducted with highly purified 26S proteasome or PA700 from bovine red blood cells.

**RESULTS**

Rpt subunits defective for ATP binding are differentially assembled into 26S proteasome. To evaluate the role of ATP binding by Rpt subunits...
in the cellular assembly of 26S proteasome, we expressed FLAG-tagged versions of individual subunits as either a wild-type or an ATP binding-defective mutant protein in HEK293 cells (Supplemental Figure 1). We compared the fate of each mutant Rpt subunit to that of its wild-type counterpart and to the fates of other mutant Rpt subunits with respect to 26S proteasome assembly. With the exception of Rpt4, we engineered and selected cells that stably expressed given FLAG-Rpt subunit pairs at levels approximately equal to one another and to the corresponding endogenous protein (Supplemental Figure 2). We were unsuccessful in generating HEK293 cells that stably expressed mutant Rpt4 at levels sufficient for further analysis because the mutant protein appeared to be unstable and was rapidly degraded after expression (data not shown). Nevertheless, mutant FLAG Rpt4 was produced at suitable levels during transient expression, and this method was used for analysis of this protein.

As an initial assessment of 26S proteasome assembly, we subjected cell extracts to glycerol density gradient centrifugation. The 26S proteasome and its separate 20S proteasome and PA700 component subcomplexes sediment at characteristic and distinguishable positions by this method (31). Thus, the distribution profile of a FLAG-tagged protein in gradient fractions reflects the protein’s steady state presence in these protein complexes. As reported previously, FLAG-tagged wild-type Rpt subunits were assembled into intact 26S proteasome, albeit with different efficiencies (39). For example, nearly all wild-type FLAG-Rpt3 and FLAG-Rpt6 were found in 26S proteasome, whereas other FLAG-Rpt proteins were found both in 26S proteasome and, to variable extents, in slower-sedimenting complexes including both intact PA700 and smaller complexes that are likely intermediates of PA700 assembly (Figure 1). ATP-binding mutations had differing effects on the incorporation of the six Rpt subunits into the 26S proteasome. Mutants of Rpt1, Rpt2, Rpt3, and Rpt4 were largely excluded from gradient fractions characteristic of 26S proteasome, and in each case accumulated in slower-sedimenting gradient fractions characteristic of intact PA700 and/or of smaller complexes (Figure 1A). In contrast to the assembly defects displayed by these mutant subunits, mutant Rpt5 was assembled into 26S proteasome and featured a distribution profile similar to that of wild-type Rpt5. Mutant Rpt6 also assembled into 26S proteasome although not as efficiently as its wild-type counterpart. Thus, although an appreciable portion of the mutant Rpt6 had a distribution profile characteristic of 26S proteasome, a significant portion was shifted to gradient fractions characteristic of PA700 (Figure 1B). In sum, these results demonstrate that Rpt subunits have different and characteristic requirements for ATP-binding for their steady-state assembly into 26S proteasome. Notably, mutant Rpt subunits defective in 26S proteasome assembly appeared to be competent for assembly into intact PA700 (see below).

**Characterization of purified protein complexes containing ATP binding-defective Rpt subunits.** To characterize the structural and functional features of complexes containing FLAG-Rpt subunits, we purified wild-type and mutant FLAG-Rpt proteins using anti-FLAG beads and then compared the properties of the resulting protein complexes. Affinity purification of each wild-type FLAG-Rpt subunit yielded structurally intact and functionally active 26S proteasomes, as judged by multiple criteria. For example, these complexes migrated on native PAGE as a mixture of singly and double-capped 26S proteasomes (Figures 2A-5A and Supplemental Figures 3 and 4), sedimented through glycerol density gradients to positions characteristic of 26S proteasomes (Figures 2C-5C), contained known component subunits of 26S proteasomes, and catalyzed peptidase and ATP-dependent protease activities diagnostic of 26S proteasomes (Figures 2B-5B).

Similar analysis of affinity-purified mutant FLAG-Rpt5, and mutant FLAG-Rpt6 demonstrated that each of these proteins also was incorporated into structurally intact 26S proteasomes (Figures 2 and 3). These results are consistent with those obtained with crude cell extracts and confirm that ATP-binding mutants of either Rpt5 or Rpt6 are competent for 26S proteasome assembly. Smaller portions of these mutant subunits were found in other complexes, including intact PA700. These complexes were obvious by distribution patterns in glycerol gradient fractions (Figure 2C and 3C), but were not well-resolved by native PAGE. Further characterization of 26S proteasomes and PA700 containing mutant Rpt5 and mutant Rpt6 is presented below.
Affinity-purification of ATP-binding mutants of FLAG-Rpts 1-4 isolated no or very low levels of intact 26S proteasome, as expected from results of glycerol density gradient centrifugation of corresponding crude cell extracts (Figures 4 and 5, and Supplemental Figures 3 and 4). Instead, these mutant FLAG Rpt subunits were associated with a heterogeneous group of protein complexes, including PA700. Comparison of the distribution profiles of individual mutant subunits on density gradient centrifugation before and after affinity chromatography, suggested that some complexes present in the initial extract were unstable and dissociated during affinity purification. For example, most mutant FLAG-Rpt 1 sedimented significantly slower after affinity-purification than before purification (Figures 1A and 5C). Likewise, the recovery of mutant FLAG Rpt2 as intact PA700 was poor after affinity purification (Supplemental Figure 4 and data not shown). We speculate that salt conditions used for elution of unbound proteins during affinity-chromatography promote dissociation of complexes with lower stabilities due to the ATP-binding mutations. In any case, these results highlight an important role for ATP binding by Rpt1-4 in formation and stability of 26S proteasome and distinguish these subunits from Rpt5 and Rpt6, whose ATP binding defects had little effect on 26S proteasome assembly and stability.

ATP-binding mutants have different effects on in vitro assembly of 26S proteasome and mimic their cellular roles. To further evaluate the significance of these distinctions among Rpt subunits for ATP binding-dependent 26S proteasome assembly, we purified intact PA700 from cells expressing Rpt subunits of each category and tested PA700 function in an in vitro 26S assembly assay with purified 20S proteasome. As expected, PA700 from cells expressing wild-type Rpt subunits activated the proteasome’s hydrolysis of peptide substrates, a functional indication of 26S proteasome assembly, and did so comparably to activation by purified bovine PA700 (Figure 6). PA700 isolated from cells expressing ATP-binding mutants of Rpt5 or Rpt6, subunits that were incorporated normally into 26S proteasome in intact cells, activated the 20S proteasome to a similar extent as PA700 containing their wild-type counterparts. In contrast, PA700 containing ATP-binding mutants of Rpt1 or Rpt3 had no detectable activating effect on proteasome activity. Thus, ATP-binding mutant Rpt subunits that were either competent or incompetent for assembly into 26S proteasome in intact cells mimicked their respective assembly competencies in this in vitro assembly assay. These results suggest that ATP binding by different individual Rpt subunits plays different roles in 26S proteasome assembly, and are consistent with a model in which cellular 26S proteasome is normally assembled by the binding of intact PA700 to 20S proteasome (see Discussion).

26S proteasome and PA700 containing a single ATP-binding mutant Rpt subunit have near-normal ATPase activities. To determine the effect of ATP binding-defective subunits on functions of proteasome complexes, we purified complexes containing wild-type and mutant Rpt subunits and compared various activities. Mutant FLAG-Rpt5 and mutant FLAG-Rpt6 were each assembled into both 26S proteasome and PA700. Proteasome complexes containing either of these mutant subunits had ATPase specific activities indistinguishable from those of complexes containing their respective FLAG wild-type subunits and from those of 26S proteasome and PA700 purified from bovine red blood cells (Table 1). Thus, the presence of a single ATP-binding defective Rpt subunit did not significantly affect the ATPase function contributed by the five remaining wild-type subunits of the complexes.

ATP-binding mutants of other FLAG-Rpt subunits did not assemble into 26S proteasome, but did assemble into PA700 to variable extents. To determine if mutant Rpt subunits defective for 26S proteasome assembly conferred different ATPase properties on intact PA700 compared to their assembly-competent mutant counterparts, we measured ATPase activity in two purified assembly-defective PA700 complexes. PA700 containing either mutant FLAG-Rpt1 or FLAG-Rpt3 had ATPase activities similar to their wild-type counterparts or to PA700 from bovine red blood cells. Collectively, these results demonstrate that the presence of a single ATP binding-defective subunit in these complexes has little effect of the ability of the five normal ATPase subunits to hydrolyze ATP and suggest that basal ATPase activity of proteasome complexes does not require a
mechanism in which Rpt subunits are obligately coupled (see below).

26S proteasomes containing a single ATP-binding mutant Rpt subunit are defective in the degradation of ubiquitylated and non-ubiquitylated substrates but not of short peptides. To further analyze effects of Rpt subunits with ATP-binding defects on functions of proteasome complexes, we compared protease activities of 26S proteasomes with wild-type or ATP-binding mutant Rpt subunits. As suggested by the initial characterization, 26S proteasomes containing either mutant Rpt5 or mutant Rpt6 featured peptidase activities comparable to those of normal 26S proteasomes (Figure 7A). These results indicate that defective ATP binding by these specific subunits has no significant effect on PA700-induced gating of the proteasome. In contrast, each mutant 26S proteasome was severely defective for the degradation of proteins such as polyubiquitylated-Sic, a model polyubiquitylated 26S proteasome substrate, and casein, a structurally-disordered protein known to be degraded by the 26S proteasome in an ubiquitin-independent fashion (Figure 7B and 7C). Thus, despite their near normal rates of ATP hydrolysis and an open substrate entry gate, 26S proteasomes harboring a single ATP binding-defective subunit were crippled for degradation of protein substrates. In the presence of proteasome inhibitors such as MG132, wild-type 26S proteasomes deubiquitylated substrates, which accumulated as unmodified proteins (Figure 7B). Deubiquitylation is catalyzed by multiple deubiquitylase subunits of PA700 (Figure 7B), but is coupled to normal 26S proteasome protease activity (20;30-32). 26S proteasomes with mutant Rpt5 or Rpt6 also deubiquitylated substrates, but did so at a modestly yet but reproducibly slower rate (Figure 7B). Collectively, these results suggest that 26S proteasome-catalyzed protein hydrolysis is linked to ATPase activity by a mechanism that requires coordinated function among all Rpt subunits.

26S proteasomes containing a single ATP-binding mutant Rpt subunit are defective in the substrate-regulated ATPase activity and gating. We previously showed that polyubiquitin stimulated peptidase and ATPase activities of 26S proteasome, suggesting a regulatory link between substrate binding and these functions (49). Here, we have extended those findings by showing that either ubiquitylated-Sic, unanchored polyubiquitin chains, or a disordered non-ubiquitylated substrate proteins such as casein also stimulated ATPase activity of normal 26S proteasome by 3-6 fold (Figure 8). Substrate-anchored or unanchored polyubiquitin chains also stimulated proteasome gating, as indicated by 3-5 fold increased peptidase activity (Figure 8B). In contrast, tightly-folded substrates refractory to degradation, such as GFP or titin127, had no effect on either activity (Figure 8D). Substrate-induced stimulation of ATPase did not appear to depend on or to be obligately-linked to proteolysis because stimulation occurred similarly with proteasomes inhibited by MG132 or epoxomicin (Figure 8E and data not shown). These results suggest that substrate binding or engagement rather than substrate hydrolysis increased ATPase activity and proteasome gating. Interestingly, protein substrates did not stimulate ATPase activity of isolated PA700 even though PA700 likely binds substrates similarly to 26S proteasome (Figure 8A). Thus, physical interaction between PA700 and 20S proteasome may be required for substrate-induced stimulation of ATPase activity. To determine the role of ATP-binding by individual Rpt subunits on substrate-induced stimulation of ATPase and peptidase activities, we repeated these experiments with 26S proteasomes containing ATP-binding mutants of either Rpt5 or Rpt6. Neither ATPase activity nor peptidase activity was stimulated by protein substrates or unanchored polyubiquitin chains in these mutant 26S proteasomes (Figures 8B,8C, and 8E). These results indicate that although 26S proteasomes with ATP binding-defective subunits have normal ATPase activity and gating properties in the absence of substrates, they are unable to regulate these features in the presence of protein substrates. Defective protein degradation by these proteasomes suggests that these regulatory features are important for normal proteasome function and likely require coordinated action among a full complement of functional Rpt subunits.

DISCUSSION

Despite their structural homology, the six Rpt subunits of the 26S proteasome appear to play different roles in multiple aspects of proteasome
function. For example, the C-termini of Rpt subunits have different roles in 26S proteasome activation and cellular assembly (38-40;51). Here we show that defective ATP binding has different effects on 26S proteasome assembly for various Rpt subunits. Several previous studies explored the role of ATP binding by Rpt subunits on cellular 26S proteasome assembly. In yeast, ATP-binding mutations of different Rpt subunits produced disparate effects on proteasome assembly and function (22). Although the general conclusions of those studies are similar to ours, the exact pattern of assembly defects for the Rpt subunits differed, perhaps because different specific mutations were used or because of differences in assembly mechanisms between yeast and mammalian cells (22). While our work was in progress, Lee et al reported the effect of ATP binding mutations on assembly of Rpt subunits into 26S proteasomes in HeLa cells (44). In contrast to our results, they reported defective 26S proteasome assembly for every mutant subunit. The basis for this discrepancy is unclear, but differences between the studies include cell lines, the methods of protein expression, and the amino acid substitutions for the mutant subunits. Of these, we consider differences in stable versus transient expression to be the most likely cause of discrepant results. In fact, we noted that mutant Rpt4 behaved differently under transient and stable expression conditions (see Results). We also observed that some mutant Rpt subunits reduced the post-assembly in vitro stability of 26S proteasome. Thus, specific details of in vitro manipulation and analysis of the mutant proteasomes may determine whether the mutant subunits are judged to have an assembly defect.

We undertook this work, in part, to evaluate the physiologic relevance of the in vitro assembly of 26S proteasome from purified 20S proteasome and PA700 (20;52). This reconstitution is strictly dependent on ATP binding, but the relative roles of individual Rpt subunits for ATP binding in this process are unknown. Furthermore, the significance of 26S proteasome formation from 20S proteasome and intact PA700 subcomplexes is uncertain because data supporting alternative models of cellular PA700 assembly into the 26S proteasome have been presented (42). One model features formation of PA700 by sequential addition of multiple PA700 subcomplexes to 20S proteasome, which serves as a required assembly template (40;53). By this mechanism, intact PA700 would not exist as an isolated cellular complex. A second model, compatible with our in vitro assembly reaction, involves formation of intact PA700 prior to its binding to 20S proteasome. Support for this model includes cellular accumulation of PA700 with Rpt subunits lacking required 20S binding elements or when 20S proteasome content is reduced by RNAi (38;39). Moreover, PA700 can be reconstituted in vitro from three subcomplexes in the absence of 20S proteasome (54). The current data provide additional support for the 20S-independent model because ATP binding-defective Rpt subunits incompetent for 26S proteasome assembly also accumulated in intact PA700 (Figure 1). Finally, we purified intact PA700 containing either wild-type or mutant Rpt subunits and demonstrated that their relative abilities to reconstitute 26S proteasome in vitro mirrored their effects on cellular 26S proteasome assembly. These results further support direct binding of intact PA700 to 20S proteasome in cells. Despite these data, an appreciable fraction of the intact PA700 containing certain mutant Rpt subunits was lost during affinity purification, perhaps by dissociation of mutant PA700 into smaller complexes. These results suggest that PA700 assembly and stability are sensitive to ATP binding and are consistent with in vitro data in which reconstitution of intact PA700 from three PA700 subcomplexes depended on ATP binding (54). Post-assembly dissociation of mutant PA700 could also account for some of the discrepancies about effects of mutant Rpt subunits noted above. Our current data do not address the molecular basis for how ATP binding by various Rpt subunits is either required or unnecessary for 26S proteasome assembly. Previous work established the role of dedicated assembly chaperones for 26S proteasome assembly, and the function of these factors may be governed by ATP binding of their client Rpt subunits (42;55).

We were surprised that 26S proteasomes or isolated PA700s containing a mutant Rpt subunit had basal ATPase specific activities similar to those of normal complexes. A recent study provided evidence for a highly concerted mechanism of proteasomal ATPase activity involving ordered cyclical ATP binding, ATP hydrolysis, and product release by pairs of Rpt subunits oriented opposite to one another in the ATPase ring (56). Ac-
Accordingly, we expected a single defective ATP-binding subunit to significantly disrupt ATPase activity. However, the near-normal ATPase activity of an Rpt ring with an ATP binding-defective subunit indicates that a highly concerted mechanism is not an absolute requirement for ATP hydrolysis and that ATP binding and hydrolysis can occur by a stochastic mechanism characteristic of certain bacterial Clp AAA protein rings (57). Likewise, the basal level of peptide hydrolysis by 26S proteasomes containing mutant Rpt subunits did not differ from wild-type proteasomes. Peptide hydrolysis is a monitor of 20S proteasome gating and is promoted upon PA700 binding (18;20). However, gating does not appear to be a simple two-state function but rather is variably modulated by factors including the status of Rpt-bound ATP and binding of polyubiquitin by the assembled 26S proteasome (35;49;58). Previous work demonstrated a role for ATP-binding by Rpt2 in gating of yeast 26S proteasome, an effect we were unable to evaluate here because of the assembly defect caused by ATP-binding mutant Rpt2 (21). Nevertheless, our results show that ATP-binding defects of either Rpt5 or Rpt6 have little effect on gating of 26S proteasomes under basal conditions and are consistent with differential roles of various Rpt subunits for regulation of proteasome function.

In contrast to their normal basal ATPase and peptidase activities, 26S proteasomes containing either mutant Rpt5 or Rpt6 were severely crippled for ATP-dependent degradation of both polyubiquitylated proteins and disordered, non-ubiquitylated proteins. These results suggest that processing of protein substrates requires the coordinated action of a full complement of functional Rpt subunits. We propose that defective proteolysis by mutant proteasomes is a consequence of an impaired regulatory response to protein substrates. Proteins susceptible to proteasomal degradation, as well as unanchored polyubiquitin chains, enhanced both ATPase and peptidase activities of normal 26S proteasome by up to 5-fold. However, neither of these effects occurred with 26S proteasomes containing mutant Rpt5 or Rpt6, suggesting that these defects were closely related to the failure of proteolysis. Thus, substrate-stimulated ATPase activity and gating appear to require, and may impose, tight coordination and cooperation among functional Rpt subunits for protein degradation. Although this type of regulation is prevented by the presence of a single ATP binding-defective subunit, the precise mechanistic relationships among these processes are unclear, and these processes may not be obligately linked. For example, degradation of non-ubiquitylated proteins requires ATP binding but not hydrolysis (18;20), indicating that degradation is enhanced by increased gating without increased ATPase activity. Likewise, substrate-activated ATPase activity does not require concomitant protein hydrolysis because it occurred in MG132-inhibited proteasomes. Finally, both ATPase activity and gating were stimulated by unanchored polyubiquitin chains, showing that each effect can be achieved in the absence of protein hydrolysis (49). It is possible that defects in substrate-regulated gating and ATPase activity, as well as in overall proteolysis, are consequences of reduced substrate binding by mutant proteasomes (35). Deubiquitylation by MG132-inhibited proteasomes was modestly but reproducibly inhibited in mutant proteasomes, an effect that also could be accounted for by reduced substrate binding. Regardless of the exact relationships among these processes, our results indicate that protein substrates promote their own degradation by enhancing proteasomal functions required for their degradation. These substrate-induced functions require concerted action among a fully-functional complement of Rpt subunits.

Quantitative comparison of activities among protein complexes depends on accurate measurements of enzymatic activities and protein content of the complexes. The assays for ATPase, peptidase, and protease activities have been validated by multiple criteria essential for quantification of enzyme activity. A larger source of possible error in these comparisons is the quantification of proteasome complex content. To achieve protein quantification necessary for normalization among samples, we isolated individual proteasome complexes by glycerol gradient centrifugation after affinity purification and then measured and compared levels of both FLAG-Rpt protein and other constituent subunits using standards of highly purified bovine PA700 and 26S proteasome (Supplemental Figure 5). This approach verified the identity of proteasome complexes and ensured that measures of their levels used for calculations were not confounded by including the content of
mixed complexes, unassembled Rpt subunits, or other proteins. The similar values of specific activities obtained for wild-type FLAG-Rpt proteasome complexes and highly purified bovine proteasome complexes provides additional confidence in the quantitative comparison of specific activities among various samples. Finally, comparison of complexes containing wild-type and mutant Rpt subunits must account for possible contributions of the corresponding endogenous wild-type Rpt subunits to measured activities of mutant complexes. This concern is not applicable to isolated PA700 or to 26S proteasomes with a single PA700 cap (which typically represented about half of the 26S proteasome content) since they should contain FLAG-tagged subunits exclusively. In contrast, doubly-capped 26S proteasomes could contain one normal and one mutant PA700. Although wild-type subunits may contribute normal activities to such “mutant” samples, the large defects in proteolysis and in substrate-stimulated ATPase and peptidase activities of these same samples indicate that mutant subunits dominate their functional features.

In summary, we have demonstrated that ATP-binding by certain, but not each Rpt subunit, is an important requirement for cellular assembly of 26S proteasome in mammalian cells. ATP binding-defective Rpt subunits severely inhibited the ability of 26S proteasomes to degrade protein substrates, possibility as a result of loss of substrate-induced activation of ATPase activity and gating. These results indicate that proteolysis by the 26S proteasome requires highly concerted function among its ATPase subunits.

FOOTNOTES

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FIGURE LEGENDS

Figure 1. ATP-binding mutants differentially affect the assembly of Rpt subunits into the 26S proteasome in HEK293 cells. Extracts of HEK 293 cells stably expressing either indicated wild-type (WT) or ATP-binding mutant FLAG-Rpt subunits were subjected to glycerol density gradient centrifugation. Gradient fractions were western blotted for FLAG protein. “20S,” “PA700,” and “26S,” with corresponding arrows indicate the peak sedimentation positions of purified bovine 20S proteasome, PA700, and 26S proteasome, respectively. Panel A. Distribution profiles of wild-type and mutant FLAG-Rpt1, FLAG-Rpt2, FLAG-Rpt3, and Flag-Rpt4. Panel B. Distribution profiles of wild-type and mutant FLAG-Rpt5 and FLAG-Rpt6. Similar results were obtained in at least three independent experiments.

Figure 2. Affinity-purification and characterization of wild-type and mutant FLAG-Rpt5 protein complexes. Extracts of HEK293 cells expressing wild-type (WT) or ATP-binding mutant (K233A) FLAG-Rpt5 were subjected to affinity purification on anti-FLAG beads. Panel A. FLAG-Rpt5 samples eluted from anti-FLAG beads were subjected to native PAGE and stained with silver (left), overlaid with Suc-Leu-Leu-Val-Tyr-AMC substrate for in-gel proteasome activity (center) or blotted with anti-FLAG antibodies (right). Panel B. WT and K233A FLAG-Rpt5 proteins were assayed for hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC. “Mock” is a sample from extracts of HEK 293 cells not expressing FLAG protein subjected to the same procedure (left). Affinity-purified WT and K233A samples were subjected to western blotting of indicated proteasome subunits (right). Panel C. WT and K233A affinity-purified samples were subjected to glycerol density gradient centrifugation. Fractions were blotted for FLAG protein and assayed for proteasome activity using Suc-Leu-Leu-Val-Tyr-AMC substrate. Known sedimentation positions of indicated proteasome complexes shown with arrows. “AS” represents applied sample.

Figure 3. Affinity-purification and characterization of wild-type and mutant FLAG-Rpt6 protein complexes. Extracts of HEK293 cells expressing wild-type (WT) or ATP-binding mutant (K196A) FLAG-Rpt6 were subjected to affinity purification on anti-FLAG beads. Panel A. FLAG-Rpt6 samples eluted from anti-FLAG beads were subjected to native PAGE and stained with silver (left), overlaid with Suc-Leu-Leu-Val-Tyr-AMC substrate for in-gel proteasome activity (center) or blotted with anti-FLAG antibodies (right). Panel B. WT and K196A FLAG-Rpt6 proteins were assayed for hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC (left). Affinity-purified WT and K196A samples were subjected to western blotting of indicated proteasome subunits (right). Panel C. WT and K196A affinity-purified samples were subjected to glycerol density gradient centrifugation. Fractions were blotted for FLAG protein and assayed for proteasome activity using Suc-Leu-Leu-Val-Tyr-AMC substrate. Known sedimentation positions of indicated proteasome complexes are indicated by shown with arrows. “AS” represents applied sample.
**Figure 4. Affinity-purification and characterization of wild-type and mutant FLAG-Rpt3 protein complexes.** Extracts of HEK293 cells expressing wild-type (WT) or ATP-binding mutant (K212A) FLAG-Rpt3 were subjected to affinity purification on anti-FLAG beads. *Panel A.* FLAG-Rpt3 samples eluted from anti-FLAG beads were subjected to native PAGE and stained with silver (left), overlaid with Suc-Leu-Leu-Val-Tyr-AMC substrate for in-gel proteasome activity (center) or blotted with anti-FLAG antibodies (right). *Panel B.* WT and K212A FLAG-Rpt3 proteins were assayed for hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC (left). Affinity-purified WT and K212A samples were subjected to western blotting of indicated proteasome subunits (right). *Panel C.* WT and K212A affinity-purified samples were subjected to glycerol density gradient centrifugation. Fractions were blotted for FLAG protein and assayed for proteasome activity using Suc-Leu-Leu-Val-Tyr-AMC substrate. “AS” represents applied sample. Known sedimentation positions of indicated proteasome complexes are shown with arrows 1.

**Figure 5. Affinity-purification and characterization of wild-type and mutant FLAG-Rpt1 protein complexes.** Extracts of HEK293 cells expressing wild-type (WT) or ATP-binding mutant (K222A) FLAG-Rpt1 were subjected to affinity purification on anti-FLAG beads. *Panel A.* FLAG-Rpt1 samples eluted from anti-FLAG beads were subjected to native PAGE and stained with silver (left), overlaid with Suc-Leu-Leu-Val-Tyr-AMC substrate for in-gel proteasome activity (center) or blotted with anti-FLAG antibodies (right). *Panel B.* WT and K222A FLAG-Rpt1 proteins were assayed for hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC in solution (left). Affinity-purified WT and K222A samples were subjected to western blotting of indicated proteasome subunits (right). *Panel C.* WT and K222A affinity-purified samples were subjected to glycerol density gradient centrifugation. Fractions were blotted for FLAG protein and assayed for proteasome activity using Suc-Leu-Leu-Val-Tyr-AMC substrate. Known sedimentation positions of indicated proteasome complexes are shown with arrows as in Figure 1.

**Figure 6. in vitro assembly of 26S proteasome from purified 20S proteasome and PA700 containing wild type or ATP-binding mutant Rpt subunits mirrors relative cellular 26S proteasome assembly properties.** PA700s containing either wild-type (WT) FLAG-Rpt subunits or corresponding ATP-binding mutant (K/A) FLAG-Rpt subunits were purified by glycerol density gradient centrifugation of anti-FLAG affinity-purified samples. Equivalent amounts of PA700, based on normalization by FLAG and Rpt2 content, were assayed for ATP-dependent activation of latent 20S proteasome activity. The peptide hydrolyzing activity of 20S proteasome in the absence of PA700 (20S) was assigned a value of 100 and activities in the presence of PA700 (+PA700) with indicated FLAG-Rpt protein are expressed as a percentage of that value. 20S proteasome activation by an equivalent amount of purified bovine PA700 (“bovine”) was determined for comparison. Data represent mean values triplicate assays ± SD. Similar results were obtained with two independent preparations of proteins.

**Figure 7. 26S proteasome complexes with a single ATP-binding mutant Rpt subunit have normal peptidase activity, but are defective in the degradation of protein substrates.** 26 proteasomes containing either wild-type (WT) or ATP-binding mutant FLAG-Rpt subunits were isolated by glycerol density gradient centrifugation after affinity purification on anti-FLAG beads, or were purified from bovine red blood cells. Equivalent amounts 26S proteasome assayed for proteasome function. *Panel A.* Hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC. Data represent mean values of triplicate assays ± SD. *Panel B.* Degradation of (Ub)_6−His-Sic in the presence (+) or absence (−) of 50 μM MG132. “Control” is substrate incubated in the absence of proteasome. “PA700” is substrate incubated with an equivalent molar content of purified bovine PA700. Western blotting was conducted with anti-His antibody. *Panel C.* Hydrolysis of [methyl-¹⁴C]casein. Each data point represents the mean value of triplicate assays ± SD. Similar results were obtained for data in each panel in at least three independent experiments.
Figure 8. 26S proteasome complexes with a single ATP-binding mutant Rpt subunit are defective in substrate-activated ATPase and peptidase activities. 26S proteasomes containing indicated wild-type (WT) or ATP-binding mutant FLAG-Rpt subunits were affinity purified on anti-FLAG beads and then isolated by glycerol density gradient centrifugation. Bovine 26S proteasome and PA700 were purified from red blood cells. Panel A. Indicated proteasome complexes were assayed for ATPase activity in the absence (Control) or in the presence of polyubiquitylated-Sic (480 nM) or casein (500 nM). Panel B. Indicated 26S proteasomes were assayed for ATPase in the absence (Control), or in the presence of polyubiquitylated-Sic (480 nM), casein (500 nM), or unanchored polyubiquitylated chains (450 nM). Panel C. Indicated 26S proteasomes were assayed for hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC hydrolyzing activity in the absence (Control) or presence of polyubiquitylated-Sic (480 nM). Panel D. Bovine 26S proteasome was assayed for ATPase activity in the absence (Con) or presence of indicated proteins (500 nM); “GFP” is green fluorescent protein; “Titin127” is the I27 domain of titin. Panel E. Indicated 26S proteasomes were assayed for ATPase activity in the absence (Control) or in the presence of polyubiquitylated Sic (480 nM) and/or MG132 (100 μM). In all panels, activity of the control was assigned a relative value of 1.0 and other activities are expressed relative to that. Each value represents the mean value ± SD of at least two independent experiments (and in most cases four independent experiments) in which each assay was performed in triplicate.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
TABLE 1.

| Proteasome Complex | ATPase activity (pmol Pi/ min/ pmol) |
|--------------------|-------------------------------------|
| **Experiment 1**   |                                     |
| 26S Bovine         | 9.8 ± 0.39                          |
| PA700 Bovine       | 9.2 ± 0.26                          |
| PA700 Rpt1 K222A   | 12.2 ± 0.04                         |
| PA700 Rpt3 K212A   | 9.8 ± 1.6                           |
| **Experiment 2**   |                                     |
| 26S Rpt6 WT        | 9.5 ± 0.59                          |
| 26S Rpt6 K196A 26S | 14.3 ± 0.56                         |
| PA700 Rpt6 WT      | 11.7 ± 0.10                         |
| PA700 Rpt6 K196A   | 15.7 ± 0.75                         |
| **Experiment 3**   |                                     |
| 26S Rpt5 WT        | 12.0 ± 0.96                         |
| 26S Rpt5 K233A     | 8.6 ± 0.21                          |
| PA700 Rpt5 WT      | 12.4 ± 0.06                         |
| PA700 Rpt5 K233A   | 11.2 ± 0.03                         |
| **Experiment 4**   |                                     |
| Rpt1 WT PA700      | 5.6 ± 0.54                          |
| Rpt1 K222A PA700   | 7.9 ± 0.48                          |

Table 1. Proteasome complexes with a single ATP-binding mutant Rpt subunit have normal basal ATPase activities. 26S proteasomes or PA700 containing either wild-type or ATP-binding mutants of indicated FLAG-Rpt subunits were isolated by glycerol-density gradient centrifugation after affinity-purification on anti-FLAG-beads, as described under Experimental Procedures. Bovine 26S proteasome and PA700 were purified from bovine red blood cells. Results for individual indicated experiments were obtained with complexes assayed concurrently. Data represent mean values of triplicate assays ± SD. Similar results were obtained with at least two independent preparations of each protein.
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