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ATP Hydrolysis-Dependent Disassembly of the 26S Proteasome Is Part of the Catalytic Cycle

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Summary

ATP hydrolysis is required for degradation of polyubiquitinated proteins by the 26S proteasome but is thought to play no role in proteasomal stability during the catalytic cycle. In contrast to this view, we report that ATP hydrolysis triggers rapid dissociation of the 19S regulatory particles from immunopurified 26S complexes in a manner coincident with release of the bulk of proteasome-interacting proteins. Strikingly, this mechanism leads to quantitative disassembly of the 19S into subcomplexes and free Rpn10, the polyubiquitin binding subunit. Biochemical reconstitution with purified Sic1, a prototype substrate of the Cdc34/SCF ubiquitin ligase, suggests that substrate degradation is essential for triggering the ATP hydrolysis-dependent dissociation and disassembly of the 19S and that this mechanism leads to release of degradation products. This is the first demonstration that a controlled dissociation of the 19S regulatory particles from the 26S proteasome is part of the mechanism of protein degradation.

Introduction

The 26S proteasome is a highly conserved protease that plays a central role in the control of protein stability in eukaryotic cells. It is the most complex example of ATP-dependent proteases, which have active sites sequestered inside a barrel-shaped core and which recruit substrates with help of a dedicated ring of ATPases (reviewed in Pickart and Cohen [2004]).

The mechanism of substrate recruitment is unique for each ATP-dependent protease, but only the 26S proteasome evolved to recognize substrates via a post-translational modification with a polyubiquitin chain (Chau et al., 1989; Thrower et al., 2000). The current view is that the timing and the specificity of ubiquitin-mediated proteolysis are mainly controlled at the step of substrate recognition by a specific ubiquitin ligase. The variety of substrate recognition mechanisms by ubiquitin ligases well reflects the large number of proteins that are targeted for proteolysis. However, in addition to the polyubiquitin chain, other factors have recently been implicated in substrate recruitment. First, many ubiquitin ligases interact with the 26S proteasome, including Ubr1 and Ufd4 (Xie and Varshavsky, 2000), SCF and APC (Verma et al., 2000), and Hul5 (Leggett et al., 2002). At least in the case of Ufd4, the interaction is direct and essential for substrate instability (Xie and Varshavsky, 2002). Second, various adaptor proteins can bind the 26S proteasome and participate in recognition of the polyubiquitin chain. These include the UbL-UBA domain proteins Rad23 and Dsk2, and the AAA-type ATPase Ufd1/Cdc48/p97. Since different adaptor proteins facilitate degradation of different substrates, the adaptor proteins may contribute to the specificity of proteolysis by a yet unknown mechanism (Verma et al., 2004).

In addition to the complex substrate recognition mechanism, the 26S proteasome has an elaborate structure. While its prokaryotic prototypes are oligomeric assemblies of two types of subunits, the 26S proteasome is composed of 33 proteins organized in two subcomplexes, the 20S proteolytic core and the 19S regulatory particle. The crystal structure of the 20S reveals four tightly stacked rings of β- and α-type subunits, with the two inner β rings having proteolytic activity (Lowe et al., 1995; Groll et al., 1997). In the 26S particles, the outer α rings of the 20S form a gating channel (Groll et al., 2000), which interacts with the base of the 19S, composed of six distinct AAA-type ATPases and two nonenzymatic subunits, Rpn1 and Rpn2 (Glickman et al., 1998; Braun et al., 1999). The remaining 11 subunits of the 19S form an asymmetrical lid. Among these, the function of only two subunits is known. Rpn10 binds the polyubiquitin chain (Deveraux et al., 1994) and recruits substrates (Verma et al., 2004; van Nocker et al., 1996). Rpn11 is a deubiquitinating enzyme (Verma et al., 2002; Yao and Cohen, 2002), which by separating the polyubiquitin chain presumably allows substrate unfolding and translocation into the proteolytic core (Braun et al., 1999; Lee et al., 2001; Navon and Goldberg, 2001; Benaroudj et al., 2003).

One of the main unresolved issues regarding the 26S proteasome is whether it functions as a stable particle or as a dynamic machine that disassembles during the catalytic cycle. On the one hand, the 19S and 20S subcomplexes dissociate in the absence of a nucleotide and reassemble in the presence of ATP (Ganoth et al.,

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1988; Eytan et al., 1989; Driscoll and Goldberg, 1990; Armon et al., 1990; Hoffman and Rechsteiner, 1997; Adams et al., 1998). Similarly, the Rpn10 polyubiquitin receptor was found as an assembled and free protein. In cell extracts, the 20S core is typically present in excess over the regulatory particles and can interact with either one or two 19S or with an alternative activator complex, the 11S (Whitby et al., 2000) or Blm 10 (Schmidt et al., 2005). These observations suggest that a controlled cycle of assembly and disassembly of the 26S particles could be a factor in protein degradation.

On the other hand, difficulties in preparation of sufficient quantities of polyubiquitinated substrates limited the attempts to address whether the 26S proteasome remains stable during the catalytic cycle. Consequently, the 26S particles were proposed to function as stable complexes based on indirect evidence. While various nucleotides can support degradation and undergo hydrolysis by the 26S proteasome, they cannot replace ATP in the formation of 26S (Armon et al., 1990). Although the molecular basis for this phenomenon is unknown, the observation was interpreted as evidence for stability of the 26S proteasome during its catalytic cycle. A potential for ATP hydrolysis-dependent dissociation was demonstrated for bacterial ClpAP protease, whose half-life was increased 5-fold in the presence of ATPγS (Sigh et al., 1999). Nevertheless, the ClpAP complex remained stable during multiple rounds of ATP hydrolysis and peptide bond cleavage. Thus, disassembly is not an obligatory step in the catalytic cycle of ClpAP, at least in assays with model substrates. Finally, analysis of the interspecies 26S hybrids formed during mixing of cell extracts revealed an exchange of subcomplexes (6%–24% per hour, Hendil et al., 2002). However, this approach also failed to firmly establish whether the exchange is linked to the catalytic cycle.

In this study, we analyzed the stability of the yeast 26S proteasome in two experimental paradigms: during ATP hydrolysis-dependent release of the bulk of endogenous proteasome-interacting proteins, and during degradation of the purified Sic1 substrate of Cdc34/SCF^{Cdc4} ubiquitin ligase. Our results suggest that a tightly controlled cycle of assembly and disassembly of the 20S, the 19S, and the interacting proteins is part of the mechanism of protein degradation.

Results

ATP Hydrolysis-Dependent Release of Endogenous PIPs from the 26S Proteasome Is Coincident with Dissociation of the 19S

We reasoned that analysis of the mechanism by which the 26S proteasome releases endogenous proteasome-interacting proteins (PIPs) is essential for understanding its function. PIPs were initially defined as a group of unrelated proteins that could be trapped in a complex with the 26S proteasome in the presence of ATPγS, either via binding to a substrate or via direct interaction with the 19S (Verma et al., 2000).

To monitor the release of PIPs from the 26S proteasome, we chose to immunopurify the complexes through the FLAG epitope-tagged Pre1 (^{F}Pre1) β-type subunit of the 20S (Verma et al., 2000). This approach held two advantages. First, in the presence of ATP, it ensured isolation of the 20S bound 19S without interfering with the 20S-19S or the 19S-PIPs interaction. Second, rapid isolation of the complexes at 0°C should allow trapping the PIPs even in the presence of ATP, while subsequent incubation at 30°C should lead to a controlled release of the PIPs from immobilized ^{F}Pre1 complexes.

Indeed, incubation of α-^{F}Pre1 IPs with ATP at 30°C led to release of multiple proteins (Figure 1A, lane 2). The release was blocked by ATPγS (Figure 1A, lane 1), indicating dependence on ATP hydrolysis. Most of these proteins were recovered in solution during the first 5 min of incubation, suggesting rapid release (Figure 1A, compare lanes 2 and 4). Mass spectrometric analysis (data not shown) revealed that this set of proteins was similar to that previously identified in ^{F}Rpt1/ATPγS samples (Verma et al., 2000). It included SCF ubiquitin ligase, which we could detect by Western blot in the ^{F}Pre1 samples prior to, but not after the incubation at 30°C (Figure 1B, lanes 1 and 3). Like other PIPs, SCF was recovered among the released components (Figure 1B, lane 2), while ATPγS blocked its release (Figure 1B, lane 4).

However, contrary to the expectation that in the presence of ATP the 19S particles remain bound to the 20S, we observed dissociation of the 19S subunits from ^{F}Pre1^{PIPs} samples (Figure 1C, lanes 1–3). The dissociation was blocked by low temperature (Figure 1C, lanes 4–6) or by ATPγS (Figure 1C, lanes 7–9), indicating dependence on ATP hydrolysis. In the presence of ATP, the majority of the released components was recovered in solution during the first 5 min of incubation, suggesting rapid release (Figure 1C, lane 1). The dissociation was limited to the 19S subunits, as we did not observe α or β subunits among the released proteins (Figures 1C and 1F, lane 4).

Two observations suggest that the ATP hydrolysis-dependent dissociation was limited to the fraction of the 26S interacting with PIPs. First, the release of the 19S was coincident with the release of the PIPs. This was most striking in analysis of the components released from ^{F}Pre1^{PIPs} (Figures 1A, 1B, 1C, and 1F, lane 4). Second, the fraction of the released 19S was proportional to the fraction of the ^{F}Pre1 complexes isolated with PIPs. This varied between 20% and 60% of total ^{F}Pre1^{PIPs} complexes that can be eluted from beads with FLAG peptide (Figure 1F, lane 1) and could be quantitatively assessed in measurements of the ^{EGFP}Rpn3-derived fluorescence. In the presence of ATPγS, the 19S visualized via ^{EGFP}Rpn3 subunit dissociated from ^{F}Pre1^{PIPs} complexes with single exponential kinetics and a half-life of about 124 min at 20°C (Figure 1D, ^{F}Pre1^{PIPs}, ATPγS). In contrast, in the presence of ATP, the half-life of ^{EGFP}Rpn3 complexes was about 8.6 min at 20°C (Figure 1D, ^{F}Pre1^{PIPs}, ATP), equivalent to a half-life of ~4.3 min at 30°C. Importantly, in about 20 min at 20°C or 10 min at 30°C, fluorescence in ^{F}Pre1^{PIPs} sample stabilized on the level of the PIP-free ^{F}Pre1 sample (Figures 1F, lane 2, and 1D, ^{F}Pre1, ATP). The stabilization was linked to a change in the dissociation kinetics, and the PIP-free ^{F}Pre1 complexes were stable in the presence of ATP despite detectable ATP hydrolysis (Figure 1E). Only the removal of the nucleotide facili-

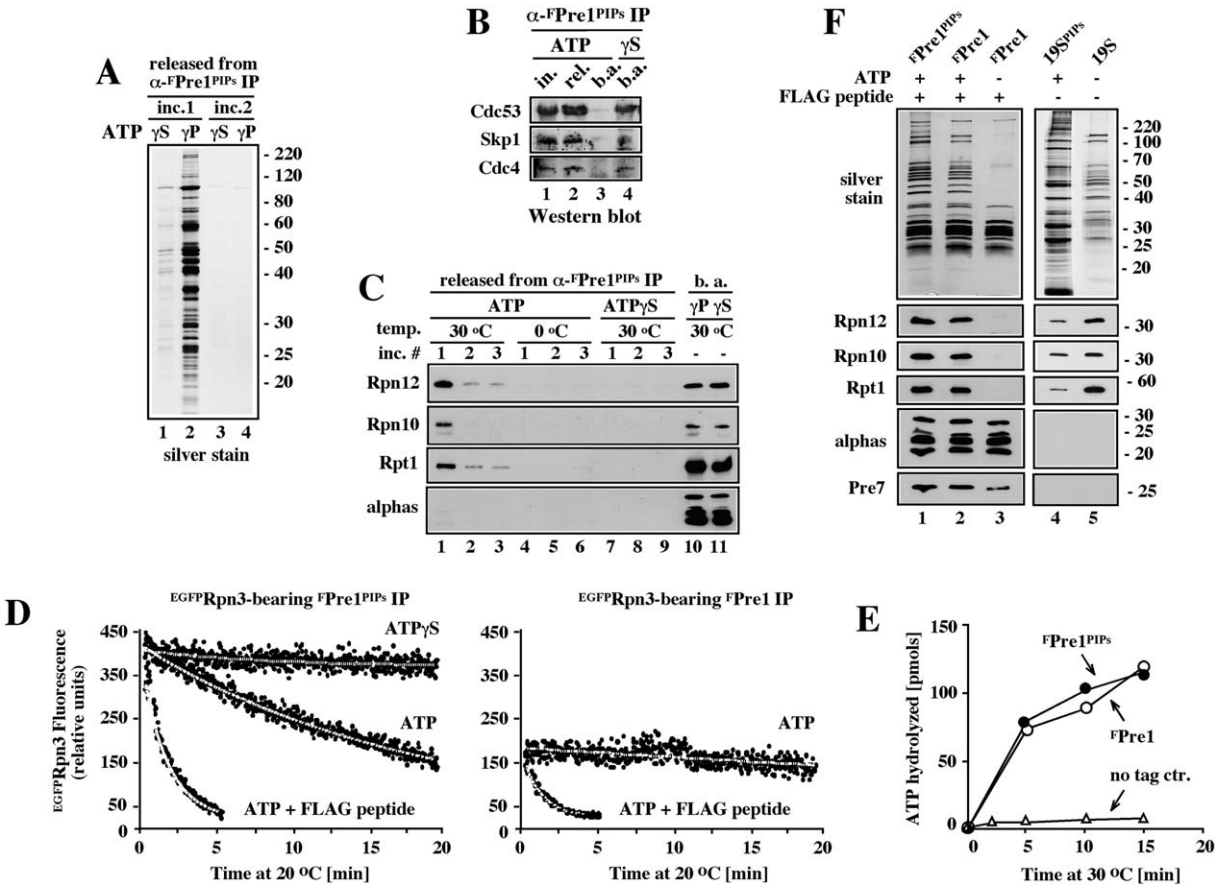

Figure 1. ATP Hydrolysis-Dependent Dissociation of PIPs Is Coincident with Dissociation of the 19S from ^{F}Pre1^{PIPs} Samples

ATP hydrolysis-dependent dissociation of PIPs (A), SCF (B), and the 19S (C). ^{F}Pre1^{PIPs} IPs were exposed to three consecutive 5 min incubations at 30°C with 500 μl of buffer U containing ATP (γP) or ATPγS (γS), as indicated. The supernatants were separated from the beads followed by precipitation of the released proteins with TCA, SDS-PAGE, and silver stain. In.: input; rel.: released; b.a.: beads after; Cdc53, Skp1 and Cdc4: subunits of SCF; Rpn12, Rpn10, and Rpt1: subunits of the 19S.

(D) Dissociation kinetics. Time course of changes in fluorescence intensity corresponding to ^{EGFP}Rpn3-bearing ^{F}Pre1^{PIPs} or ^{F}Pre1 complexes on beads was analyzed at the flow cytometer in the presence of ATPγS, ATP, or ATP and FLAG peptide, as indicated. Data represent duplicate measurements.

(E) ATP hydrolysis in the ^{F}Pre1 samples. ATPase assays were performed with eluted ^{F}Pre1^{PIPs} (filled circles), ^{F}Pre1 (open circles), or with a "no tag" control sample (Δ). The assays contained ~3 pmol of the 19S distributed between doubly and singly capped proteasomes.

(F) Comparison of samples prepared from ^{F}Pre1^{PIPs} IPs. SDS-PAGE and WB analysis of ^{F}Pre1 complexes eluted with FLAG peptide prior to (^{F}Pre1^{PIPs}, ATP+) or after (^{F}Pre1, ATP+) PIPs removal by three consecutive 5 min incubations at 30°C or after subsequent incubation without nucleotide for 30 min at 30°C (^{F}Pre1, ATP–). Lanes 4 and 5: the released 19S^{PIPs} and the 19S. Because of low protein content, the 19S^{PIPs} sample was visualized by silver stain that was overdeveloped. Western blots were exposed equally for all samples.

tated formation of the 20S (Figure 1F, lane 3) and the 19S (Figure 1F, lane 5) from the PIP-free ^{F}Pre1 sample.

Thus, the 19S present in the PIP-free ^{F}Pre1 sample was part of a classical 26S complex, which remained stable during multiple rounds of ATP hydrolysis but dissociated in the absence of a nucleotide. In contrast, the fraction of PIP-enriched ^{F}Pre1^{PIPs} complexes released both the PIPs and the 19S as result of ATP hydrolysis.

The 19S and SCF Released from the ^{F}Pre1^{PIPs} Samples in an ATP Hydrolysis-Dependent Manner Are Disassembled into Subcomplexes and/or Subunits

As part of characterization of the ATP hydrolysis-dependent disassembly of the 26S particles, we used HPLC to analyze the size of the 19S complexes sequentially released from the ^{F}Pre1 samples: first, as a result of ATP hydrolysis (the 19S^{PIPs}; Figure 1F, lane 4) and second, as a result of nucleotide depletion (the 19S; Figure 1F, lane 5).

Surprisingly, the 19S^{PIPs} complex was quantitatively dissociated into free subunits (Figure 2A, top) or subcomplexes reminiscent of the lid, the base, and free Rpn10 (Figure 2A, bottom). Dilution likely played a role in preventing reassociation of the released components because reformation of subcomplexes was observed even upon a mild, 2-fold concentration of the 19S^{PIPs} sample prior to HPLC (Figure 2A, bottom), while a 4-fold concentration restored the size of intact 19S (data not shown). Strikingly, the Skp1 subunit of SCF

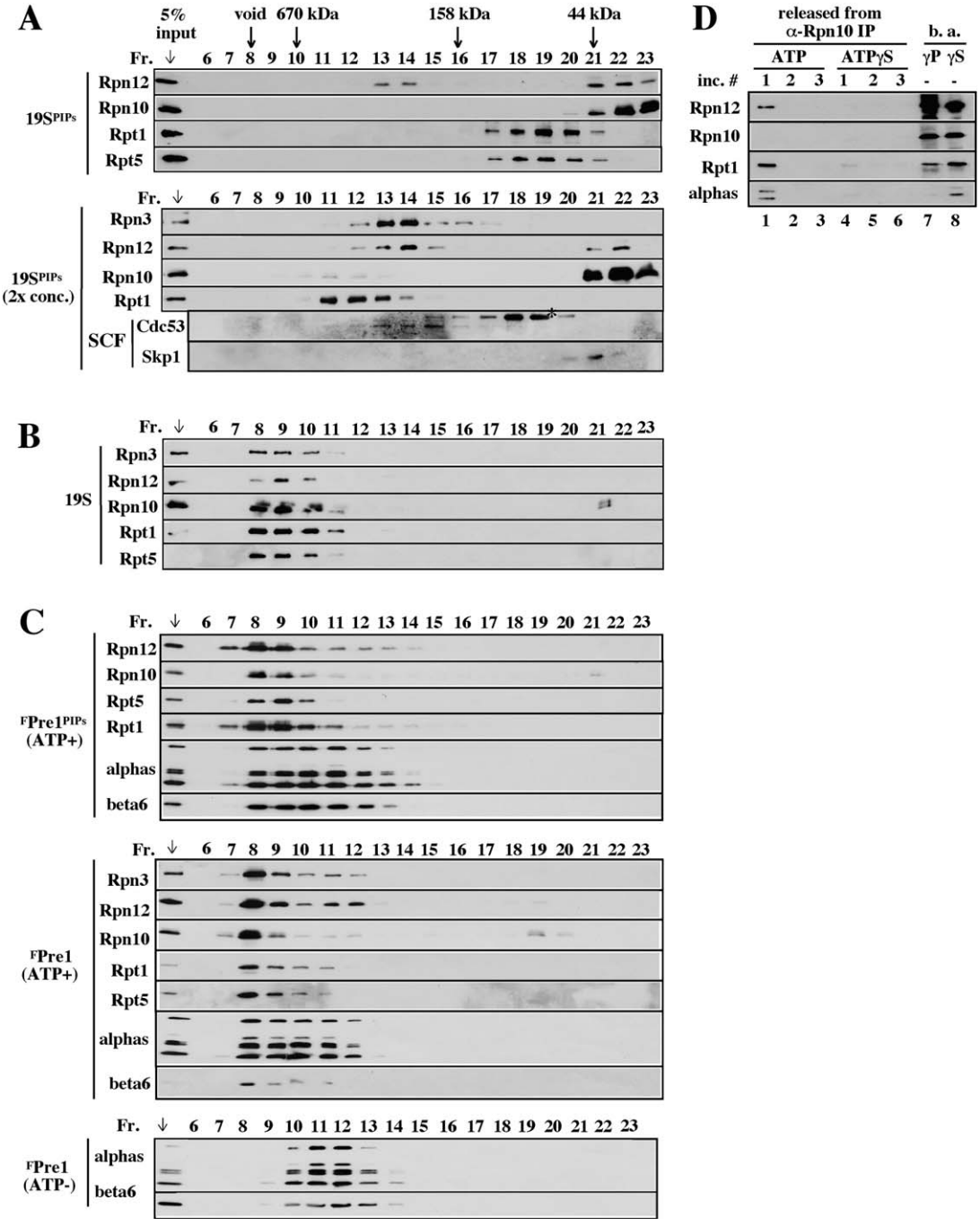

Figure 2. ATP Hydrolysis-Dependent Dissociation Has a Powerful Disassembling Effect on the 19S^{PIPs} and SCF

(A) The 19S^{PIPs} sample contains subcomplexes or free subunits. The 19S^{PIPs} sample was prepared by incubating ^{F}Pre1^{PIPs} samples immobilized on 100 μl of α-FLAG agarose with 500 μl of buffer U with ATP for 5 min at 30°C followed by analysis of supernatants by HPLC on Superdex 200^{HR} either immediately (top) or after 2-fold concentration on Centricon10 at 4°C (bottom).

(B) The 19S sample contains intact particles. The 19S sample was prepared by incubation of the remaining PIP-free ^{F}Pre1 complexes with 500 μl of buffer U without ATP for 30 min at 30°C, followed by analysis of supernatants by HPLC.

(C) ^{F}Pre1^{PIPs}(ATP+), ^{F}Pre1(ATP+), and ^{F}Pre1(ATP–) complexes remain stable during HPLC. Experiment as in (A) except that with ^{F}Pre1 complexes eluted from beads with FLAG peptide prior (^{F}Pre1^{PIPs}, ATP+), after (the ^{F}Pre1, ATP+) release of PIPs, or after dissociation of the 19S (the ^{F}Pre1, ATP–).

(D) ATP hydrolysis-dependent dissociation of α-Rpn10 IPs. Experiment as in Figure 1C, except that IP was done with α-Rpn10 antibodies.

present in the 19S^{PIPs} sample eluted separately from the Cdc53 subunit of SCF (Figure 2A, bottom, SCF, compare Fr. 21 with 15). Thus, SCF complexes, which remain tightly bound in assays without the 26S proteasome (Deffenbaugh et al., 2003), were disassembled upon release from the 26S. In contrast, the 19S released from the PIP-free ^{F}Pre1 complex as result of nucleotide depletion was a stable particle that included Rpn10 (Figure 2B). Similarly, the 19S present in the initial ^{F}Pre1^{PIPs} and the ^{F}Pre1 samples also eluted in intact particles (Figure 2C). Thus, the disassembly of the 19S^{PIPs} sample was not a result of the chromatography.

We confirmed the controlled dissociation of Rpn10 subunit in experiments with α-Rpn10 IPs (Figure 2D). In this case, Rpn10 protein was immobilized on beads via direct interaction with the antibodies, and, consequently, ATP hydrolysis led to the release of the remaining 19S and 20S subunits (Figure 2D, lane 1). In contrast, no protein release was observed from the α-Rpn10 IPs in the presence of ATPγS (Figure 2D, lanes 4–6).

Thus, the ATP hydrolysis-dependent dissociation of the 26S^{PIPs} proteasomes leads to release of Rpn10 subunit and to disassembly of the 19S into subcomplexes. Strikingly, this process also led to the disassembly of SCF, one of the proteasome-interacting complexes.

Quantitation of Particles Visualized by Electron Microscopy Confirms ATP Hydrolysis-Dependent Disassembly of the 26S Complexes

To gain insight into the structural changes and quantitative transitions associated with the presence of PIPs and the ATP hydrolysis-dependent disassembly, we analyzed the ^{F}Pre1^{PIPs} (Figure 1F, lane 1) and ^{F}Pre1 (Figure 1F, lane 2) samples by electron microscopy.

Both samples were a heterogeneous mixture of singly capped, doubly capped, and uncapped 20S, with a small percentage of two novel 20S structures, the 20S+ and the 20S@ (Figures 3A and 3B). This heterogeneity was expected because immunoprecipitation via the ^{F}Pre1 subunit led to isolation of all forms of 20S present in yeast extracts. Visual inspection of averaged images did not reveal major structural differences among each type of particle. The only difference was a reduced sharpness of the 19S caps in the ^{F}Pre1^{PIPs} doubly capped structures (Figure 3B). However, digital analysis in the form of difference maps identified a number of variable regions (Figure 3C, low-threshold). The largest differences were located in multiple areas at the periphery of the 19S caps (Figure 3C, high-threshold). These changes could reflect either the binding of diverse PIPs to distinct domains of the 19S or an increased conformational variability of the ^{F}Pre1^{PIPs} sample.

Visual classification of particles (14,199 total) in multiple experiments was consistent with disassembly of a fraction of the 26S structures in ^{F}Pre1^{PIPs} samples (Figure 3D). In the presence of ATPγS (Figure 3D, "before" disassembly, black bars), the ^{F}Pre1^{PIPs} samples were enriched in the doubly capped 26S structures (30.0%) when compared with the final ^{F}Pre1 preps eluted from beads after release of PIPs (Figure 3D, white bars, 16.0%). A partial enrichment was observed even in the presence of ATP (Figure 3D, dark gray bars, 24%), which could not be prevented from hydrolysis during preparation of EM grids at room temperature. Conversely, the PIP-free ^{F}Pre1 samples were enriched in the 20S structures (Figure 3D, white bars, 33%) when compared with the initial ^{F}Pre1^{PIPs}/ATPγS preps (Figure 3D, black bars, 4%). In contrast, the singly capped 26S structures were similarly represented in all preparations (44.0%–50.0%). These data suggest two possible mechanisms for ATP hydrolysis-dependent disassembly of the 26S proteasomes. The first is a coupled dissociation of both 19S from the doubly capped particles, leading to formation of uncapped 20S. In this scenario, singly capped proteasomes are presumed stable and free of PIPs. Alternatively, both doubly capped and singly capped 26S particles may interact with PIPs and dissociate in an independent stochastic manner, leading to accumulation of single capped and uncapped 20S particles as the respective products.

Consistent with the prediction that a free ring of ATPases is one of the products of the ATP hydrolysis-dependent disassembly (Figure 2A, bottom), electron microscopic analysis revealed a distinct structure of a ring in the ^{F}Pre1^{PIPs} samples (Figure 3B, "ATPase ring?"). Quantitation of particles (Figure 3D) showed that this ring structure was 15 times more abundant in the ^{F}Pre1^{PIPs} samples analyzed in the presence of ATP (3.0% of total particles) than in the presence of ATPγS (0.2% of total particles) or in the final ^{F}Pre1 preps (0.2%). This enrichment may be explained by ATP hydrolysis-dependent disassembly during adsorption to the EM grid. Interestingly, the ring did not display hexagonal symmetry and its diameter (150 ± 2 Å) was significantly larger than the diameter of the end view of the 20S particles (120 ± 2 Å), and of the base within the 26S particles (100–110 Å). If the ring does correspond to the base of the 19S, this difference may suggest that the ATP hydrolysis-dependent dissociation of the 19S is linked to a major conformational change in the ATPases. Since the six ATPases form a tight complex with two noncatalytic subunits Rpn1 and Rpn2 (Glickman et al., 1998), these subunits may contribute to the symmetry of the base structure.

In the SCF Ubiquitin-Ligase Pathway, Substrate Degradation Is Required for Coupling ATP Hydrolysis to Disassembly of the 26S Proteasome

To test directly whether substrate degradation has a role in coupling ATP hydrolysis to dissociation of the 26S, we asked whether we could induce dissociation of the PIP-free proteasomes (as shown in Figures 1F, lane 2, 1D, right, and 3A, right) by supplementing them with purified in vitro polyubiquitinated Sic1 substrate. Sic1 is an inhibitor of the yeast S phase Clb5/Cdc28 CDK and one of the best characterized naturally unstable proteins. Polyubiquitinated Sic1 remains in a tight complex with Clb5/Cdc28 (Verma et al., 2001) and SCF^{Cdc4} (Deffenbaugh et al., 2003). Thus, it differs from nonphysiological model substrates, which are not assembled with other proteins, either because they are denatured (loosely folded casein denatured ovalbumin) or polyubiquitinated without a specific E3 (lysozyme, DHFR, Ub-Pro-β-gal).

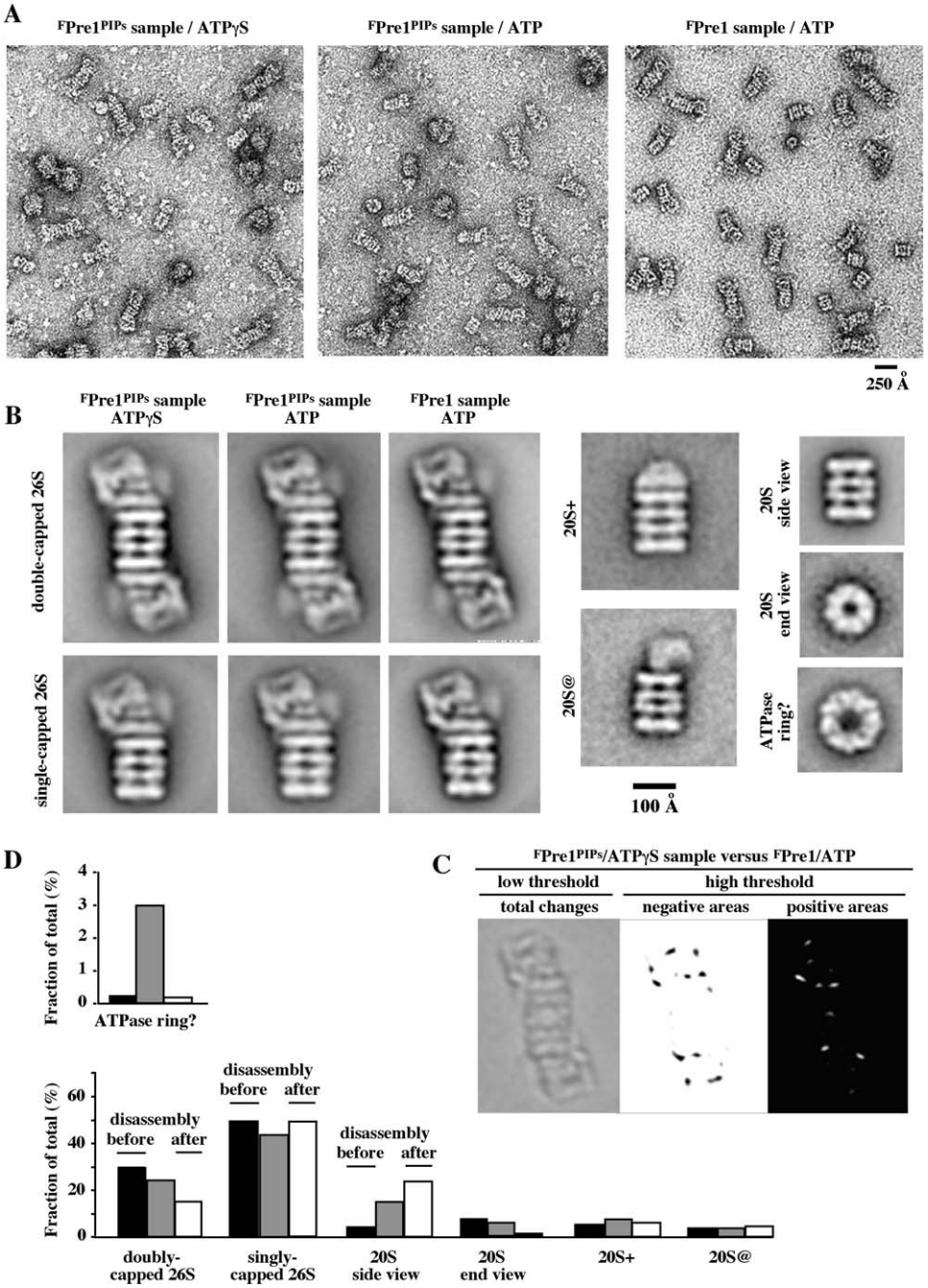

Figure 3. Electron Microscopic Analysis of Structures Present in FPre1 Samples

(A) Representative fields of the FPre1PIPs/ATPγS, FPre1PIPs/ATP, and the FPre1/ATP samples. Note that the FPre1PIPs samples contain unidentified structures and some finer material that may represent individual proteins or protein complexes.

(B) Averaged structures. Left: averaged images of the singly and doubly capped structures include at least 1000 individual particles found in each of the samples. Right: less-numerous classes of particles (20S side and end views, 20S+, 20S@, and rings) were selected from both FPre1 and FPre1PIPs preparations and contained 100–500 particles per image.

(C) Difference maps. Averages of the doubly capped structures with and without PIPs were compared digitally, using low and high threshold. Negative and positive areas indicate the position of biggest differences between the FPre1PIPs and FPre1 structures.

(D) Quantitation of particles. Bars show quantitative representation of each type of structure calculated as a percentage of the total number of particles. FPre1PIPs/ATPγS sample (black; 100% = 4103 particles), FPre1PIPs/ATP sample (dark gray; 100% = 2238 particles), and the FPre1/ATP sample (light gray; 100% = 7858 particles).

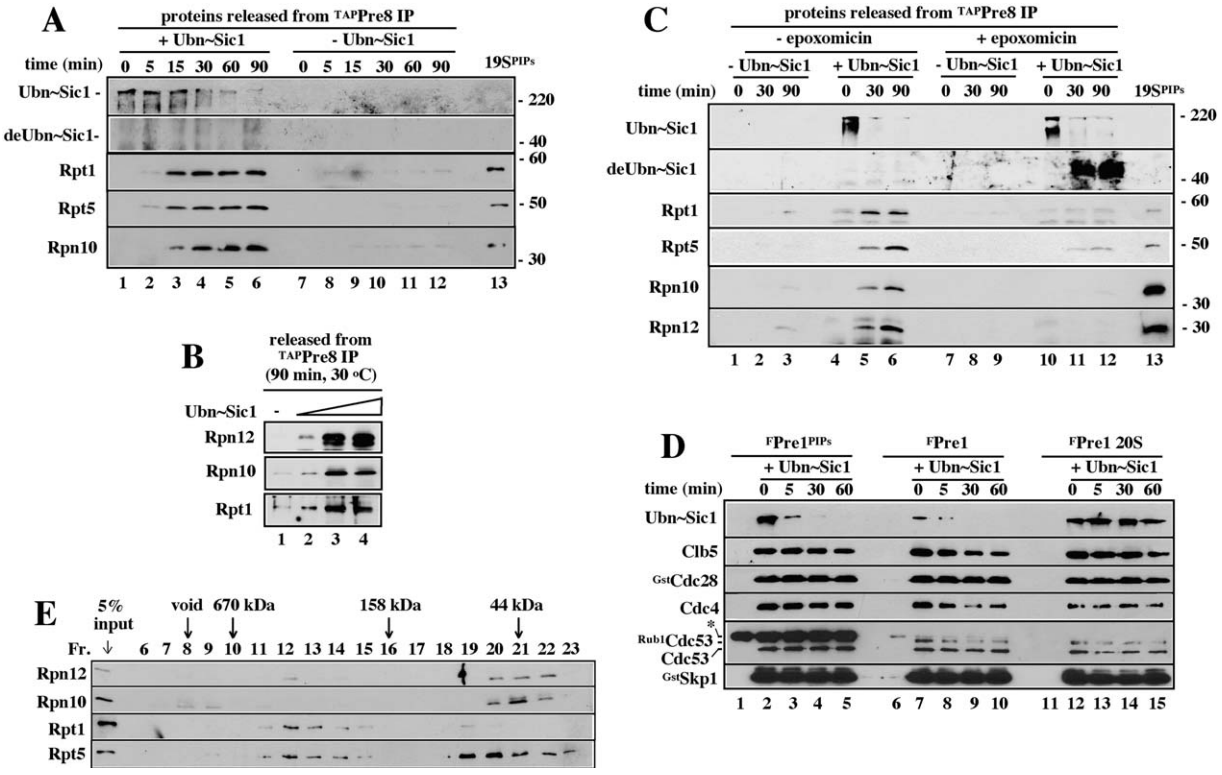

Figure 4. ATP Hydrolysis-Dependent Dissociation of 26S Complexes during Degradation of Purified Polyubiquitinated Sic1

(A) Degradation of Sic1 is coincident with dissociation of the 19S. TAPPre8 proteasomes (~4 pmol) immobilized on 10 μl beads were incubated at 30°C with or without 2 pmol of polyubiquitinated Sic1 (Ubn~Sic1) in 30 μl of a complete ubiquitination mix, followed by SDS-PAGE/WB of the released material. DeUbn~Sic1: deubiquitinated Sic1. Lane 13: reference of the 19SPIPs.

(B) Titration of polyubiquitinated Sic1. Experiment like in (A), except that with different amounts of polyubiquitinated Sic1 (0, 2, 10, and 20 pmol).

(C) Requirement for intact proteolytic sites. Experiment like in (A), except that, where indicated, the proteolytic sites of the TAPPre8 complexes were inhibited with epoxomicin prior to substrate addition (Verma et al., 2002).

(D) The FPre1 and FPre1PIPs samples have similar activity in Sic1 degradation. Degradation assays as in (A) except that the FPre1 complexes, as indicated, were eluted from beads with FLAG peptide and the reactions were additionally analyzed for substrate-associating proteins. Asterisks mark irrelevant protein crossreacting with α-Cdc53.

(E) Disassembly of the 19S components. Supernatants from ten individual Sic1 degradation assays as described in (A) were combined and separated at 4°C by HPLC, as in Figure 2.

Degradation of polyubiquitinated Sic1 (Ubn~Sic1, MW > 220 kDa) by immobilized PIP-free proteasomes coincided with dissociation of the 19S, as judged by the release of the Rpt1, Rpt5, Rpn10, and Rpn12 subunits (Figures 4A, lanes 1–6, and 4C, lanes 4–6). We did not recover deubiquitinated Sic1 protein under these conditions (Figure 4A, deUbn~Sic1, lanes 1–6), confirming that disappearance of polyubiquitinated Sic1 reflected its degradation. The fraction of released 19S was proportional to the amount of degraded Sic1 (Figure 4B), with saturation at about 20 pmol. This would be equivalent to about 2–3 pmol of a substrate per 1 pmol of the 26S, assuming that the singly and doubly capped proteasomes were equally active in the degradation of Sic1 and that together they represented ~65% of total FPre1 complexes (Figure 3D).

Two observations suggest that in the absence of substrate degradation, ATP hydrolysis did not lead to dissociation of the 26S. In the absence of Sic1, no 19S subunits were released by the catalytically active (Figures 4A, lanes 7–12, and 4C, lanes 1–3) or epoxomicin-inhibited (Figure 4C, lanes 7–9) proteasomes. Release of the 19S was also undetectable when polyubiquitinated Sic1 was added but could not be degraded because of inhibition of the proteoytic sites (Figure 4C, lanes 10–12). Strikingly, in the absence of proteolysis, Sic1 accumulated as a deubiquitinated protein outside 26S particles (Figure 4C, deUbn~Sic1, lanes 10–12). This suggests that the proteasome either did not unfold Sic1 or that Sic1 protein was unfolded, yet escaped being permanently trapped within the proteolytic core.

Dissociation of the 26S proteasomes during degradation of Sic1 was linked to disassembly of the 19S into free subunits or subcomplexes (Figure 4E). This recapitulates what we observed during the ATP hydrolysis-dependent dissociation of the bulk of endogenous PIPs (Figure 2A). In both cases, the exact pattern of disassembly varied depending on the volume used to trap the released components and how the sample was reconcentrated prior to HPLC. The only consistent difference was that the kinetics of the 19S release in the reconstituted system (Figure 4A) was ~3 times slower

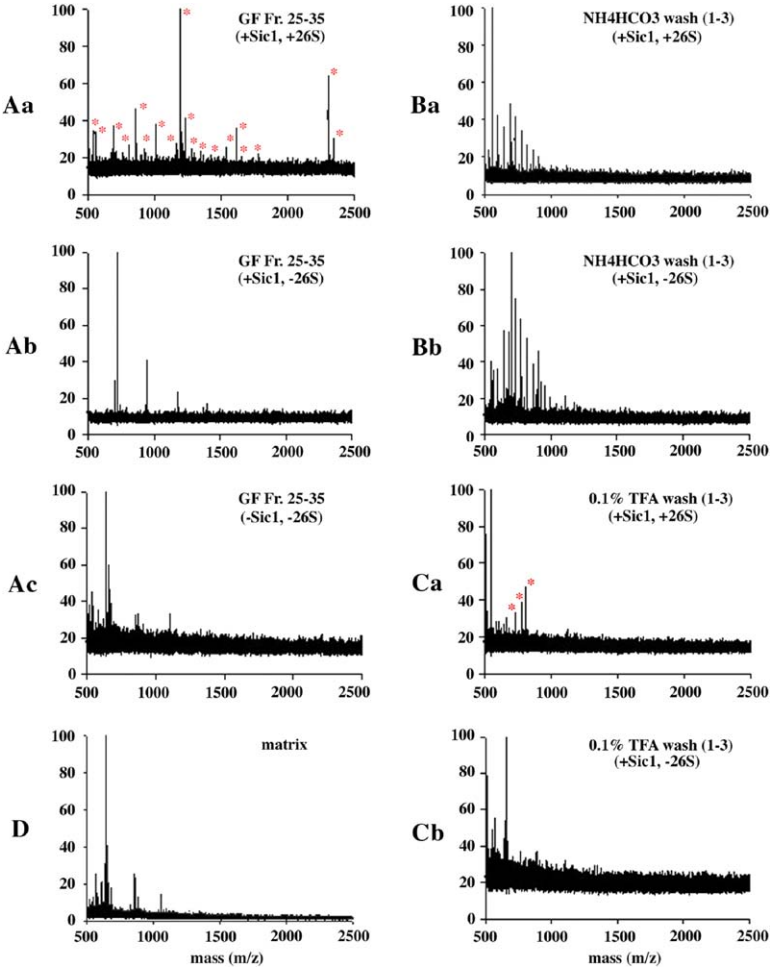

Figure 5. Mass Spectrometric Analysis of Peptides Found Inside and Outside of ^{F}Pre1 Complexes after the ATP Hydrolysis-Dependent Disassembly

Peptides present in degradation assay with 20 pmol of Sic1/Clb5/Cdc28/^{Gst}SCF^{Cdc4} and ~40 pmol of immobilized ^{F}Pre1 proteasomes were recovered in three pools.

(A) Peptides in supernatants additionally separated by HPLC on Superdex 200^{HR} (GF fractions 25–35, MW 2.0–0.5 kDa).

(B) Peptides in a bicarbonate wash of the remaining ^{F}Pre1 complexes (three washes with 100 mM ice-cold NH_4HCO_3).

(C) Peptides recovered during incubation with 0.1% TFA (three 5 min incubations at RT). Peptides in each of these fractions were analyzed as described in the Experimental Procedures.

Experiment 1 (Aa, Ba, and Ca): peptide patterns obtained in assay with Sic1 and the ^{F}Pre1 complexes (+Sic1, +26S). Experiment 2 (Ab, Bb, and Cb): peptide patterns with Sic1 and α-FLAG beads without ^{F}Pre1 (+Sic1, –26S). Additional controls (Ac): peptide patterns in supernatants from α-FLAG beads alone (–Sic1, –26S).

(D) Background pattern of matrix.

Asterisks indicate most prominent peptides present only in Sic1 degradation assay (+Sic1, +26S) but not in controls. Each experiment was repeated three times without major changes in the overall patterns.

than in the presence of endogenous PIPs (Figure 1C). A technical difficulty in the recruitment of polyubiquitinated Sic1 to the immobilized proteasomes was likely the rate-limiting step because when eluted from beads, the same ^{F}Pre1 samples degraded Sic1 more rapidly (Figure 4D, lanes 7–10), with a rate similar to that of the ^{F}Pre1^{PIPs} samples (Figure 4D, lanes 2–5). In both cases, only the Sic1 protein was rapidly degraded, while the Sic1-associated proteins Clb5, Cdc28, Cdc4, Cdc53, and Skp1 either escaped degradation or were degraded with a slower rate.

Thus, using purified Sic1 substrate of the Cdc34/SCF^{Cdc4} ubiquitin ligase, we recapitulated the key aspects of the mechanism by which the 26S proteasome dissociates during the release of the bulk of endogenous PIPs.

ATP Hydrolysis-Dependent Dissociation of the 19S Is Coincident with Release of Degradation Products

To test directly whether degradation products were released or remained trapped inside the 20S after the controlled disassembly of the 26S particles, we performed mass spectrometric analysis of peptides recovered from Sic1 degradation assay with PIP-free ^{F}Pre1 complexes.

The most distinct pattern of peptides was detected among the components released from the ^{F}Pre1 samples (Figure 5Aa). This pattern was not observed in control experiments, when Sic1 was incubated with α-FLAG beads alone (Figure 5Ab) or when the supernatants were collected from α-FLAG beads without Sic1 (Figure 5Ac). In subsequent bicarbonate washes, similar patterns of peptides were observed regardless of whether the beads did or did not contain ^{F}Pre1 complexes (Figures 5Ba and 5Bb). Thus, under these conditions, we likely released unrelated peptides from the antibodies. Only a small pool of product peptides was recovered during incubation of the remaining ^{F}Pre1 samples with 0.1% of trifluoroacetic acid (TFA), which should lead to opening of the 20S (compare Figures 5Ca and 5Cb).

Comparison of the peptide patterns suggests that the ATP hydrolysis-dependent disassembly of the 26S particles coincided with the release of most product peptides.

Discussion

In this study, we provide the first experimental evidence for a controlled, ATP hydrolysis-dependent disassembly of the 26S proteasome and link it to the degradation

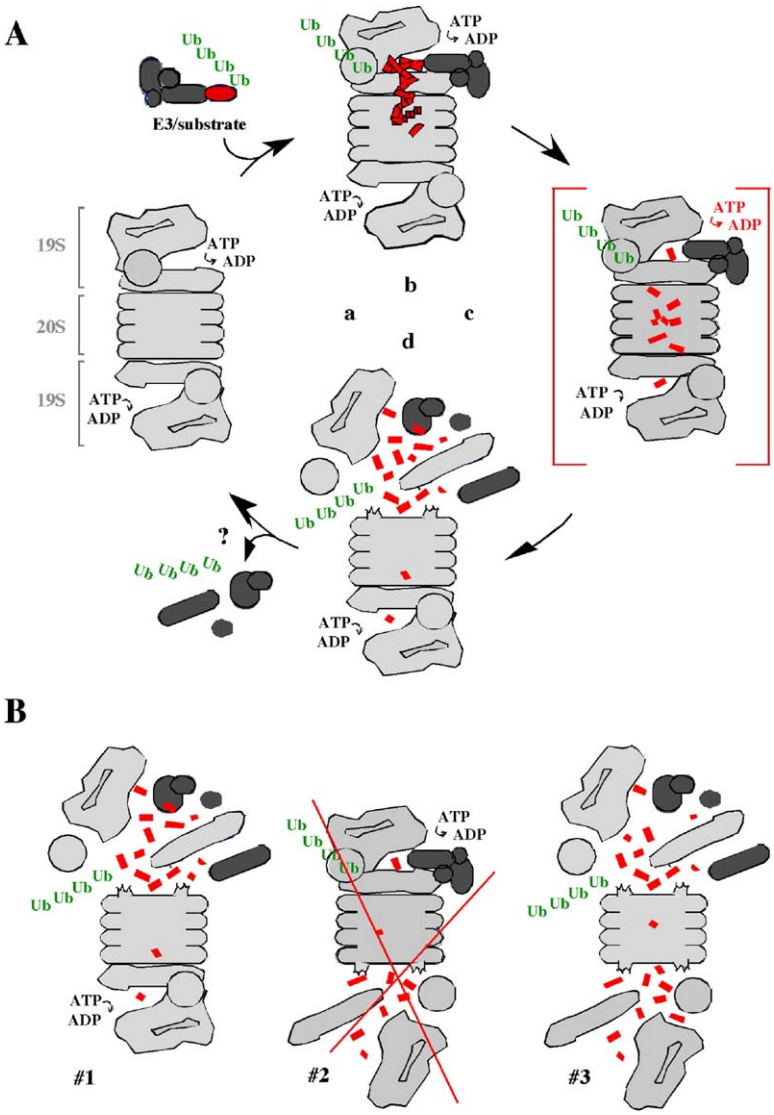

Figure 6. The "Chew and Spew" Model for Coupling ATP Hydrolysis to Disassembly of the 26S Proteasome

For simplicity, the model focuses only on the doubly capped proteasomes.

(A) The catalytic cycle. (a) Purified 26S particle hydrolyzes ATP but remains stable. (b) The 26S particle remains stable during recruitment of a polyubiquitinated substrate (red) in a complex with the E3 (dark gray). (c) Substrate degradation triggers the coupling mechanism (red brackets symbolically mark the coupling event with red ATP→ADP transition indicating the critical hydrolysis reaction). (d) The coupling mechanism generates a powerful mechanical force, likely linked to a conformational change in the ATPases, which leads not only to dissociation of the 19S but also to its disassembly into subcomplexes. This process is associated with release and disassembly of the E3 and with a burst-type "spew-like" product peptides release. The 19S components reassociate into the 26S particle, resetting the machinery into a new round of degradation.

B. Possible schemes for the disassembly. (1) Dissociation of the E3 bound 19S, with a singly capped proteasome as a product. In this scheme, the 19S-free end of the singly capped proteasome could facilitate accelerated release of product peptides, essentially as proposed by Kisselev et al. (2002) and Kisselev et al. (2003), based on model derived from 11S-20S-19S hybrid particles. (2). Disassembly of the E3-free 19S. This scheme is unlikely because it could not explain release of the E3. (3). Disassembly of two 19S caps, as a result of positive cooperativity between the 20S ends (Adams et al., 1998, Hutschenreiter et al., 2004).

of a substrate. Our data are inconsistent with the view that the 26S proteasome functions as a stable particle. Instead, they indicate that a tightly controlled cycle of assembly and disassembly of the 20S, the 19S, and the interacting proteins occurs during protein degradation. The "chew and spew" model for the catalytic cycle of the 26S proteasome summarizes this view (Figure 6).

The key feature of the proposed mechanism is the coupling between ATP hydrolysis and disassembly of the 26S particles. Clearly, in the catalytic cycle of the 26S proteasome, not every round of ATP hydrolysis leads to disassembly. We observed the controlled dissociation of the 19S only under two experimental situations: first, during the ATP hydrolysis-dependent release of the bulk of endogenous PIPs, possibly linked to degradation of copurified substrates, and second, in assays with purified polyubiquitinated Sic1 substrate of the Cdc34/SCF^{Cdc4} ubiquitin ligase. A combination of semiquantitative and qualitative evidence links the dissociation of the 26S complexes to the degradation step. This includes the stoichiometric relationship between the number of degraded substrate molecules and the number of disassembled 26S particles and the similarity between the half-life of the 26S complexes and the half-life of polyubiquitinated Sic1 (~4.3 min at 30°C). Strikingly, when the epoxomicin-inhibited 26S proteasomes were incubated with polyubiquitinated Sic1, the 26S particles remained stable even though Sic1 was separated from the polyubiquitin chain, confirming functional recruitment. Thus, the early steps in substrate recruitment preceding deubiquitination are insufficient to trigger the disassembly of the 26S. Rather, the disassembly depends on some of the later steps, which are either functionally linked to or at least very well correlated with degradation.

What could serve as a trigger for coupling ATP hydrolysis to dissociation of the 19S? The appearance of product peptides could induce a conformational change in the adjacent ATPases by inducing an allosteric change within the 20S. Indeed, analysis of the proteasome-associated ATPase HslU has confirmed the potential of AAA-type ATPases to change conformation

in a nucleotide-dependent manner (Wang et al., 2001). In addition, several studies have suggested that allosteric transitions play a role in function of the proteasome (Osmulski and Gaczynska, 2002; Hutschenreiter et al., 2004; Kisselev et al., 1999; Kisselev et al., 2002; Kisselev et al., 2003). Especially interesting are the effects associated with presence of hydrophobic peptides, which have been proposed to bind to several noncatalytic sites in the 20S and to accelerate proteolysis by inducing two types of changes. First is to allosterically activate the catalytic sites, leading to a more effective "bite and chew" degradation (Kisselev et al., 1999; Kisselev et al., 2002). Second is to accelerate the release of product peptides by promoting an opening in the α rings (Kisselev et al., 2002). The role of this mechanism is thought to be functionally similar to the role of the 11S activator (Whitby et al., 2000), which acts by opening of the 19S-free end of the proteasome. A similar role could be assigned to the ATP hydrolysis-dependent dissociation of the 19S. This mechanism not only generates the 19S-free end of the proteasome, but it was also linked to the quickest type of degradation typical of naturally unstable proteins and to the release of the product peptides. An important requirement for efficiency of such a degradation mechanism would be rapid reassociation of the 26S particles, necessary to reset the machinery for the next catalytic cycle. The reassociation was not apparent in our assays because we took precautions to prevent the reassembly (suspending 10 μl of beads in 50–500 μl of buffer guaranteed a 5- to 50-fold dilution). Nevertheless, reassembly would not be prevented by dilution under normal reaction conditions. Indeed, we observed reassembly of the released components upon reconcentration and found that the reconstituted complexes stimulated peptidase activity of the 20S, implicating functional interaction (data not shown).

A second model predicts that product peptides are required, but not sufficient, and that the coupling mechanism depends on a change induced directly in the 19S. Such a change could be induced in the ATPases in response to translocation of the substrate or to interaction with some "coupling" factor recruited with the substrate. Clb5/Cdc28 CDK and the SCF^{Cdc4} are good candidates for such a "coupling" factor in our assays. Both factors remain in a complex with polyubiquitinated Sic1 (Verma et al., 2001; Deffenbaugh et al., 2003) and interact with the 26S proteasome (Kaiser et al., 1999; Verma et al., 2000). Interestingly, endogenous SCF interacts with the 26S proteasome even when thermal inactivation of the *cdc34-1* mutant protein blocks ubiquitination (Verma et al., 2000). If substrate ubiquitination plays no role in SCF recruitment to the 26S proteasome, its degradation may be insufficient for SCF release. In such a case, direct interaction with the E3 may play a role in coordinating release of the degradation products with disassembly of the 26S and their own release.

Does the ATP-hydrolysis-mediated disassembly of the 26S proteasome depend on the function of a single "master" ATPase or reflect a coordinated activity of all six ATPases? Genetic analysis in yeast first suggested individualized roles of proteasomal ATPases by demonstrating distinct phenotypes associated with inactivation of the conserved ATP binding motif (Rubin et al., 1998). Since then, inactivation of the Rpt2 ATPase alone has been shown to inhibit the opening of the gating channel (Rubin et al., 1998; Kohler et al., 2001). In contrast, a crosslinking approach has shown that the Rpt5 ATPase interacts with a substrate-attached polyubiquitin chain, suggesting a role in substrate recruitment (Lam et al., 2002). Finally, the Rpt1 and Rpt6 ATPases were shown to bind Ubr1 and Ufd4 E3s, implying a role in recruitment of ubiquitination machineries (Xie and Varshavsky, 2000; Xie and Varshavsky, 2002). Although it is unknown which ATPases are involved in substrate unfolding and translocation to the proteolytic core, the hypothesis that the individual ATPases have specialized functions becomes increasingly attractive. In this view, the coupling between ATP hydrolysis and disassembly of the 19S could depend on a single ATPase. On the other hand, it is hard to imagine that the machinery operates without coordinating the ATPase activities, especially if they have the potential to induce disassembly of the 19S and thus interrupt proteasomal function if not controlled.

Regardless of the precise mechanism of the controlled disassembly, the most striking finding in this report is the formation of the 19S subcomplexes and subunits during the catalytic cycle. Subcomplexes of the 19S particles were previously observed only when purified *rpn10Δ* 26S mutant proteasomes were exposed to high salt concentrations (Glickman et al., 1998). This is not the case in our assays. Although ^{F}Pre1 proteasome has a FLAG epitope tag fused to the Pre1 β-type subunit (Verma et al., 2000), congruent results with the ^{TAP}Pre8, α-Rpn10, and α-Rpn12 immune complexes eliminate the possibility of an artifact. Additionally, the dependence on both ATP hydrolysis and substrate degradation demonstrates that the disassembly was highly regulated.

Interestingly, proteolysis-independent functions of the 19S or its subcomplexes have recently been implicated in nucleotide excision repair (Gillette et al., 2001), transcriptional elongation (Ferdous et al., 2001), transcriptional activation (Gonzales et al., 2002), and chromatin remodeling (Eshkova and Tansey, 2004). These results were puzzling because the 19S and its subcomplexes are normally undetectable as free particles. One possibility is that the relatively high background of proteolysis present in the conditional mutants of the proteasome could have been sufficient to generate the subcomplexes by the mechanism proposed here. In this case, the function of the 19S and its subcomplexes would be nonproteolytic, but their formation would be linked to proteolysis. Alternatively, disassembly of the 26S could be also facilitated by a yet undefined proteolysis-independent mechanism. In any case, we showed that the 26S proteasome is indeed a direct source of a powerful chaperone-like activity, which can disassemble and/or remodel the substrate-associating proteins. A clue as to why disassembly of the SCF could be beneficial comes from analysis of Cand1/Cul1 complex (Goldenberg et al., 2004). Cand1 is a negative regulator of SCF, which acts by sequestering unassembled Cul1 subunit. Thus, a mechanism that can generate free subunits from otherwise stable SCF complexes could be

necessary to allow transitions between the functional and inhibited states.

Experimental Procedures

Antibodies

We used the following antibodies: IgG Sepharose and α-FLAG M2 agarose (Sigma), rabbit α-^{Gst}Rpn10, α-^{Gst}Rpn12, α-^{Gst}Pre7, α-^{MBP}Sic1, α-^{Gst}Skp1, α-^{Gst}Cdc4, α-Cdc53 (this work; Skowyra et al., 1997; M. Goebl), rabbit α-Rpt1, α-Rpt5, and monoclonal α-alphas (Affinity Research Products, Ltd., United Kingdom), α-GFP (Clontech). Antibodies detection was by ECL (Amersham).

Yeast Strains

Yeast strains used were the following: RJD 1144—*MAT***a** *his3Δ200 leu2-3,112 lys2-801 trpΔ63 ura3-52 PRE1^{FH}::YIplac211* (*URA3*) (Verma et al., 2000); YE1350—RJD1144 + pEGFP-*RPN3::TRP1* (a pRS314-derived plasmid, this work); SC0355—*MAT***a** *ade2 arg4 leu2-3,112 trp1-289 ura3-52, Pre8-TAP* (*URA3*) (Gavin et al., 2002).

Yeast Extracts

Yeast extracts (5–10 mg/ml proteins) were prepared by grinding cells harvested at the logarithmic phase of growth on synthetic medium and blast-frozen in a 1:0.7 ratio of E buffer (50 mM Tris [pH 7.5], 20 mM $MgCl_2$, 5 mM ATP or ATPγS, 0.2 mM DTT, 10% glycerol) followed by thawing and centrifugation.

Immunopurification of ^{F}Pre1^{PIPs} and ^{F}Pre1 Proteasomes

α-FLAG agarose (10 μl) was tumbled for 1 hr at 4°C with 1 mg of ^{F}Pre1 yeast extract, followed by three washes with 1 ml of ice-cold U buffer (50 mM Tris [pH 7.5], 50 mM KCl, 5 mM ATP, 10 mM $MgCl_2$, 0.2 mM DTT), resulting in immobilized ^{F}Pre1^{PIPs} preparations. The PIP-free ^{F}Pre1 samples were prepared from ^{F}Pre1^{PIPs} samples by three consecutive 5 min incubations at 30 °C in 500 μl of buffer U. Where indicated, the complexes were eluted with FLAG peptide (5 mg/ml, 2 min at 0°C).

Preparation of the 19S^{PIPs} and the 19S

The 19S^{PIPs} and the 19S samples did not contain epitope-tagged subunits except for ^{EGFP}Rpn3, where indicated, and were isolated exclusively via interaction with the ^{F}Pre1 or ^{TAP}Pre8.

HPLC

HPLC was performed with Superdex 200 HR 10/30 column (Amersham) in buffer U at 4°C, with a flow rate of 0.5 ml/min and fraction size 500 μl, followed by protein precipitation with 10% TCA, SDS-PAGE, and Western blot.

ATPase Assay

Two picomoles of the ^{F}Pre1^{PIPs} or ^{F}Pre1 samples were equilibrated and eluted with 20 μl of 100 μM ATP in buffer U, followed by addition of 0.1 μCi of [γ-^{32}P]ATP (4500 Ci/mmol, ICN) and incubation at 30°C. After the times indicated, 2 μl of each sample was separated on PEI-cellulose in 1M formic acid, 1M LiCl (1:1), and the radioactivity of ^{32}Pi was measured in scintillation counter. The amount of ATP hydrolysis was not permitted to exceed 10% of the initial amount of ATP.

Polyubiquitination and Degradation of Sic1

Ubiquitination was performed for 1 hr at 30°C in 20 μl reactions containing 2 pmol of purified Sic1/Clb5/^{Gst}Cdc28 substrate complex, 2 pmol of ^{F}SCF^{Cdc4} or ^{Gst}SCF^{Cdc4}, 70 pmol of Cdc34ΔC, 1 pmol of Uba1, and 1.3 nmol of Ub (Deffenbaugh et al., 2003). For degradation, the mixtures were supplemented with 4–8 pmol of ^{F}Pre1 complexes as indicated and incubated at 30°C.

Identification of Proteins by Mass Spectrometry

SDS-PAGE-resolved proteins were digested with trypsin, batch fractionated on a RP micro-tip, and the peptide mixtures analyzed by MALDI-reTOF mass spectrometry (Winkler et al., 2002).

Mass Spectrometric Analysis of Peptide Patterns

Peptides in each of fraction described in Figure 5 were pooled, lyophilized, resuspended in 50 μl of 0.1% TFA, concentrated on C18 Ziptip (Millipore), eluted with 1 μl of 0.1% TFA/50% Acetonitrile saturated with α-Cyano-4-hydroxycinnamic acid matrix (Agilent) and analyzed by MALDI-MS.

Electron Microscopy

Fresh ^{F}Pre1^{PIPs} or ^{F}Pre1 samples were stained with 2% uranyl acetate on carbon-coated EM grids. Images were recorded with a JEOL 1200EX electron microscope and digitized with a Eurocore Hi-Scan film scanner at a resolution of 30 microns, or 5 Å on the specimen. Images of individual particles were aligned by a reference-independent alignment procedure using the SPIDER software (Frank et al., 1996). The procedure was repeated 6–12 times for each data set. To calculate difference maps, the averages were high- and low-pass Fourier filtered and scaled to minimize deviations.

Flow Cytometry Measurements

^{EGFP}Rpn3 ^{F}Pre1 yeast extracts with 2 mM ATPγS were incubated with 150,000 of α-FLAG beads (Buranda et al., 1999) for an hour at 4°C, followed by wash with buffer U containing 1 mM ATPγS. Ten microliter aliquots of bead suspensions were diluted to 1ml of buffer U with 1 mM ATPγS or 10 mM ATP at 20°C and analyzed on the flow cytometer immediately (shown) or at equilibrium (after one hour at RT; data not shown but used for kinetic fitting). The raw data were processed as described in Deffenbaugh et al. (2003).

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