Molecular Cell, Vol. 10, 495–507, September, 2002, Copyright ©2002 by Cell Press

Multiple Associated Proteins Regulate Proteasome Structure and Function

David S. Leggett,[1] John Hanna,[1] Anna Borodovsky,[2] Bernat Crosas,[1] Marion Schmidt,[1] Rohan T. Baker,[3] Thomas Walz,[1] Hidde Ploegh,[2] and Daniel Finley[1,4]
[1]Department of Cell Biology
[2]Department of Pathology
Harvard Medical School
Boston, Massachusetts 02115
[3]Molecular Genetics Group
John Curtin School of Medical Research
Australian National University
Canberra ACT 0200
Australia

Summary

We have identified proteins that are abundant in affinity-purified proteasomes, but absent from proteasomes as previously defined because elevated salt concentrations dissociate them during purification. The major components are a deubiquitinating enzyme (Ubp6), a ubiquitin-ligase (Hul5), and an uncharacterized protein (Ecm29). Ecm29 tethers the proteasome core particle to the regulatory particle. Proteasome binding activates Ubp6 300-fold and is mediated by the ubiquitin-like domain of Ubp6, which is required for function in vivo. Ubp6 recognizes the proteasome base and its subunit Rpn1, suggesting that proteasome binding positions Ubp6 proximally to the substrate translocation channel. *ubp6*Δ mutants exhibit accelerated turnover of ubiquitin, indicating that deubiquitination events catalyzed by Ubp6 prevent translocation of ubiquitin into the proteolytic core particle.

Introduction

The ubiquitin-proteasome pathway regulates a wide variety of biological processes (Hershko and Ciechanover, 1998). The proteasome degrades proteins conjugated to ubiquitin and thus plays a central role in this pathway (Finley, 2002). The two major subcomplexes of the proteasome are the 670 kDa proteolytic core particle (CP, or 20S particle) and the 900 kDa regulatory particle (the RP, also known as PA700 and the 19S particle) (Figure 1A). The RP is thought to bind ubiquitin chains (Lam et al., 2002), unfold the attached substrate protein, and translocate the substrate into the CP (Finley, 2002). CP subunits are arranged into four seven-membered rings, the outer rings being composed of α subunits, and the inner rings containing the proteolytically active β subunits (Groll et al., 1997). The proteolytic sites of the CP face a sealed internal chamber (Groll et al., 1997), access to which is regulated by a gated axial channel consisting of the N-terminal tails of the α subunits (Whitby et al., 2000; Groll et al., 2000; Köhler et al., 2001).

An eight-subunit subcomplex of the RP, the lid, can be dissociated from proteasomes in vitro (Glickman et al., 1998b) (Figure 1A). A second subcomplex, the base, contacts the CP and is thought to direct translocation of substrates into the CP. The base contains the six proteasomal ATPases (Glickman et al., 1998b) and appears responsible for the protein unfolding function of the RP (Braun et al., 1999). An ATPase subunit of the base, S6′/Rpt5, is critical for recognition of ubiquitin chains (Lam et al., 2002), while another ATPase, Rpt2, is required for opening of the CP channel (Köhler et al., 2001). The lid is closely related to the COP9 signalosome complex, which regulates photomorphogenesis (Glickman et al., 1998a; Wei et al., 1998) and is required for removal of a ubiquitin-like protein from cullin subunits of the SCF ubiquitin-ligase (Lyapina et al., 2001).

The proteasome has traditionally been viewed as a discrete complex containing stoichiometric subunits that survive stringent purification methods. Although conventionally purified proteasomes are relatively homogeneous, a variety of proteins interact detectably with the proteasome. Among these are ubiquitin-like proteins such as Rad23 (Schauber et al., 1998), ubiquitinating enzymes (Xie and Varshavsky, 2000; Verma et al., 2000, Tongaonkar et al., 2000), deubiquitinating enzymes (Lam et al., 1997; Papa and Hochstrasser, 1999; Verma et al., 2000), adaptor proteins (Jager et al., 2001), and cell-cycle regulators (Kaiser et al., 1999). Recently, mass spectrometry and two-hybrid screens have been used to survey proteins that interact with proteasomes (Verma et al., 2000, 2001; Ho et al., 2002; Gavin et al., 2002; Davy et al., 2001; Cagney et al., 2001). Taken together, these studies have suggested that a diverse group of proteins interacts with the proteasome. However, the functional significance of these interactions remains for the most part poorly understood, and the results obtained using different methods are not in close agreement. In some cases it is unclear whether interactions identified through screens are also seen with intact proteasomes, and in other cases it is unclear whether a significant fraction of proteasomes are associated with a given factor.

In this study, we identify proteins associated with affinity-purified proteasomes from *S. cerevisiae*. While many proteins may associate with proteasomes transiently or in low amounts, only three—Ecm29, Hul5, and Ubp6—appear to be major components. A clear role in regulating proteasome activity was observed for Ecm29 and Ubp6. Ecm29 enhances the stability of the proteasome, whereas Ubp6 contributes to its enzymatic activity. In addition, proteasomes dramatically activate Ubp6. Our results suggest that Ubp6 may help to release ubiquitin from proteasome-bound conjugates, thus preventing translocation of ubiquitin onto the CP. For many protein complexes, such as polymerases, molecular chaperones, ribosomes, cytoskeletal fibers, and nuclear pores, loosely associated cofactors are critical for function. Our results suggest that the proteasome, as previously defined, represents a core complex of salt-resistant subunits that is functionally distinct from the complex responsible for protein degradation in vivo.

[4]Correspondence: daniel_finley@hms.harvard.edu

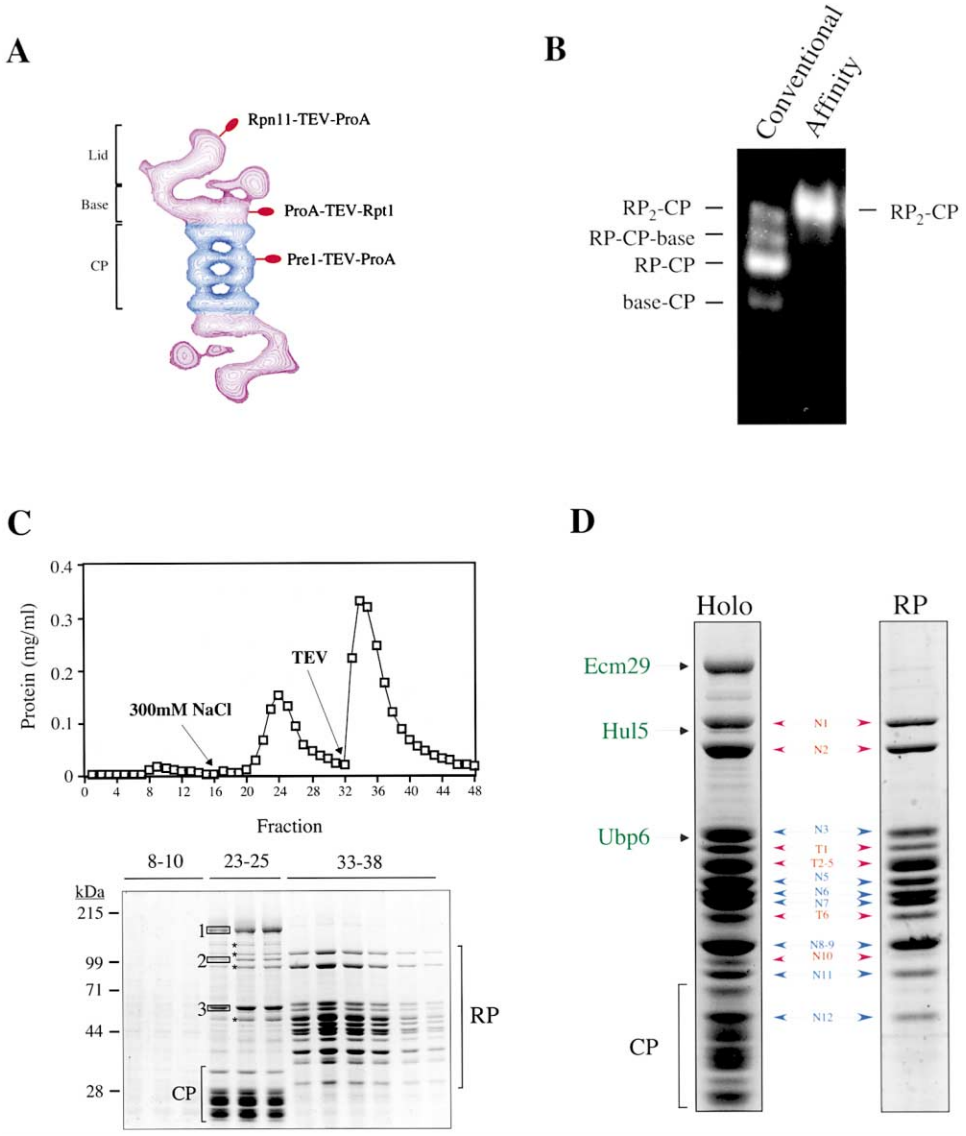

Figure 1. Salt-Released Components of the Proteasome

(A) Schematic representation of proteasome affinity tags used in this study.

(B) Proteasomes (1 μg/lane) were purified by alternative methods, separated by nondenaturing PAGE, and visualized by soaking the gel in fluorogenic substrate (LLVY-AMC), followed by UV illumination. Affinity-purified proteasomes are presumably in the RP_2CP form. The conventional preparation was according to Glickman et al. (1998a).

(C) Rpn11-TEV-ProA proteasomes were bound to IgG resin and washed with 100 mM NaCl, followed by 300 mM NaCl. Material remaining on the column was released with TEV protease. Proteins from peak fractions were analyzed by 10% SDS-PAGE and visualized with Coomassie. Bands 1–3 were identified by mass spectrometry. Bands marked by asterisks were as follows: from top to bottom, a breakdown product of Ecm29 produced by heating in the presence of SDS (also seen in Figures 2 and 3), Rpn1, Rpn2, and apparently Rpt2-5.

(D) Proteins from affinity-purified holoenzyme (Holo) and RP were analyzed by 10% SDS-PAGE and visualized with Coomassie. Components of the base are indicated in red, the lid in blue. Ubp6 migrates more rapidly than Rpn3, but the two bands are often not resolved.

Results

Composition of Affinity-Purified Proteasomes

Existing methods for proteasome purification do not allow rapid isolation of proteasomes in high purity and yield. For this purpose, we appended TEV-protease-cleavable, Protein A-derived tags to various proteasome subunits (Figure 1A; see Supplemental Data at http://molecule.org/cgi/content/full/10/3/495/DC1). Tagged subunits were expressed from their endogenous promoters to ensure synthesis at a near-physiological level. To allow for the purification of proteasome subassemblies, the base, lid, and CP were each individually tagged (Figure 1A). We initially purified proteasome holoenzymes using the Rpn11-TEV-ProA strain. Proteasomes were captured from cell extracts using IgG resin, then specifically released using the TEV endoprotease. Nondenaturing gel electrophoresis revealed a single major species having proteasome-specific peptidase activity. Surprisingly, this band migrated more slowly than that of

conventionally purified proteasomes (Figure 1B). Thus, affinity-purified proteasomes may contain major components not present in conventional samples.

Conventional methods of proteasome purification generally involve fractionation on ion-exchange resins, and therefore, exposure to high salt. To examine the effect of salt on proteasome composition, we added a salt-wash step prior to TEV elution. Three hundred millimolar NaCl released the CP together with a small number of proteins, numbered 1–3 in Figure 1C. These proteins were identified by mass spectrometry as Ecm29, Hul5, and Ubp6, respectively (see Supplemental Data at http://molecule.org/cgi/content/full/10/3/495/DC1). These proteins have been previously described (Verma et al., 2000; Lussier et al., 1997; Wang et al., 1999; Park et al., 1997), and according to genome-wide transcript profiling studies, they are coregulated with proteasome subunits (see Supplemental Data at http://molecule.org/cgi/content/full/10/3/495/DC1). A comparison of salt-washed (Figure 1D, right) and nonsalt-washed samples (Figure 1D, left) confirmed Ecm29, Hul5, and Ubp6 as major salt-released components, and suggested that Ecm29 and Ubp6 (Figure 1C) may be present in stoichiometric amounts, as judged by Coomassie staining.

To confirm the association of Ecm29, Hul5, and Ubp6 with the proteasome, TEV-eluted samples were analyzed by gel filtration chromatography. Ubp6 (Figure 2A), Ecm29 (Figure 2B), and Hul5 (Figure 2C) cofractionated with proteasome subunits as well as proteasome-specific peptidase activity. In each case, these proteins were missing from proteasomes purified from the corresponding deletion mutants (Figures 2B, 2C, and 4B). Related experiments suggested that Ubp6, Ecm29, and Hul5 bind to the proteasome independently of one another (data not shown). While Hul5 and Ubp6 are known components of the ubiquitin pathway, Ecm29 is not, and its identification as a proteasome component was surprising because it had previously been implicated in cell wall biogenesis (Lussier et al., 1997). Using mutants constructed in this and other laboratories, in which the entire coding sequence of *ECM29* is deleted, we were unable to confirm the cell wall phenotype attributed to *ecm29*Δ mutants.

Ecm29 Tethers the CP to the RP

Conventionally purified proteasomes are unstable in the absence of ATP (Hendil et al., 2002). To our surprise, it was possible to affinity purify proteasomes without nucleotide (Figure 3A). In contrast, ATP was required to recover proteasomes from an *ecm29*Δ strain (Figure 3A, top). The RP was efficiently recovered from *ecm29*Δ extracts when ATP was not included in purification buffers (Figure 3A, bottom; data not shown). Thus, the absence of Ecm29 leads to dissociation of the CP and RP when ATP is not provided. In the presence of ATP, proteasomes could be purified from the *ecm29*Δ strain; however, the *ecm29*Δ proteasomes differed from those of wild-type in that the proportion of RP_2CP was significantly reduced, consistent with a partial dissociation of the RP and CP (Figure 3A). Thus, the ATPases of the RP, which are thought to be in direct contact with the CP, appear to function redundantly with Ecm29 to stabilize the RP-CP association. Consistent with the possibility that Ecm29 is a stoichiometric component, no proteasomes resistant to the effect of *ecm29*Δ were observed.

The experiment of Figure 3A suggested that Ecm29 may function to tether the CP and the RP. A prediction of this model is that Ecm29 is competent to bind both the RP and the CP. To test the tethering model, we initially analyzed salt eluates from an IgG column loaded with Rpn11-TEV-ProA proteasomes (equivalent to fractions 23–25 of Figure 1C). The sample was resolved by gel filtration with NaCl omitted. The Ecm29 peak was coincident with that of the CP, indicating complex formation (Figure 3B). Electron micrographs of free Ecm29 reveal a V-shaped morphology in which the angle between the two domains is not strictly fixed (Figure 3C). Ecm29 complexed with the CP frequently displayed an open V-shaped morphology as well (Figure 3C, lower right). In these images, Ecm29 appeared to bind to the CP via the tip of one domain. Binding appeared to be to the outer (α) ring of CP subunits, and Ecm29 projected from the CP in the same direction as the RP, suggesting that CP-bound Ecm29 could contact the RP. In other cocomplexes (Figure 3C, lower left), the Ecm29 mass was closer to the CP, and ran transversely along the cylinder end. These complexes may correspond to the closed-angle form (Figure 3C, upper left) of free Ecm29.

To test whether Ecm29 can also bind the RP, a complementary experiment was carried out with CP-tagged proteasomes. Immobilization of the CP on IgG resin allowed for coelution of Ecm29 and RP with 300 mM NaCl. When this sample was then subjected to gel filtration in the absence of NaCl (as in Figure 3B), cofractionation of Ecm29 and the RP was observed (Figure 3D). Cofractionation was not fortuitous, since it was eliminated by 300 mM NaCl (data not shown). The ability of Ecm29 to bind both CP and RP may be sufficient to explain its role in stabilizing the holoenzyme. That Ecm29 functions in association with the proteasome in vivo is suggested by the sensitivity of *ecm29*Δ mutants to canavanine, an amino acid analog, when crossed to mutations in other proteasome-associated proteins (M.S., unpublished data).

Ubp6 Is the Predominant Ubiquitin-AMC-Hydrolyzing Activity of the Yeast Proteasome

The presence of Ubp6 in proteasomes at levels comparable to those of known subunits raised the possibility that Ubp6 may have an unrecognized role in the mechanism of ubiquitin-protein conjugate breakdown by the proteasome. To assess the contribution of Ubp6 to proteasomal deubiquitinating activity, we incubated proteasomes with [^{125}I]-ubiquitin C-terminal vinyl sulfone ([^{125}I]-UbVS), a reagent that covalently modifies the active sites of at least 6 of 17 ubiquitin hydrolases in yeast (Borodovsky et al., 2001). Affinity-purified proteasomes contain a UbVS-labeled protein of an apparent M_r of ~66 kDa, consistent with an adduct between ubiquitin (8.6 kDa) and Ubp6 (57 kDa). Proteasomes appear to be highly enriched in the 66 kDa protein, as compared to cell extracts (Figure 4A). The proteasomal UbVS target was assigned as Ubp6 based on its electrophoretic comigration with modified Ubp6 from cell extracts (Figure 4A) and its absence from proteasomes prepared

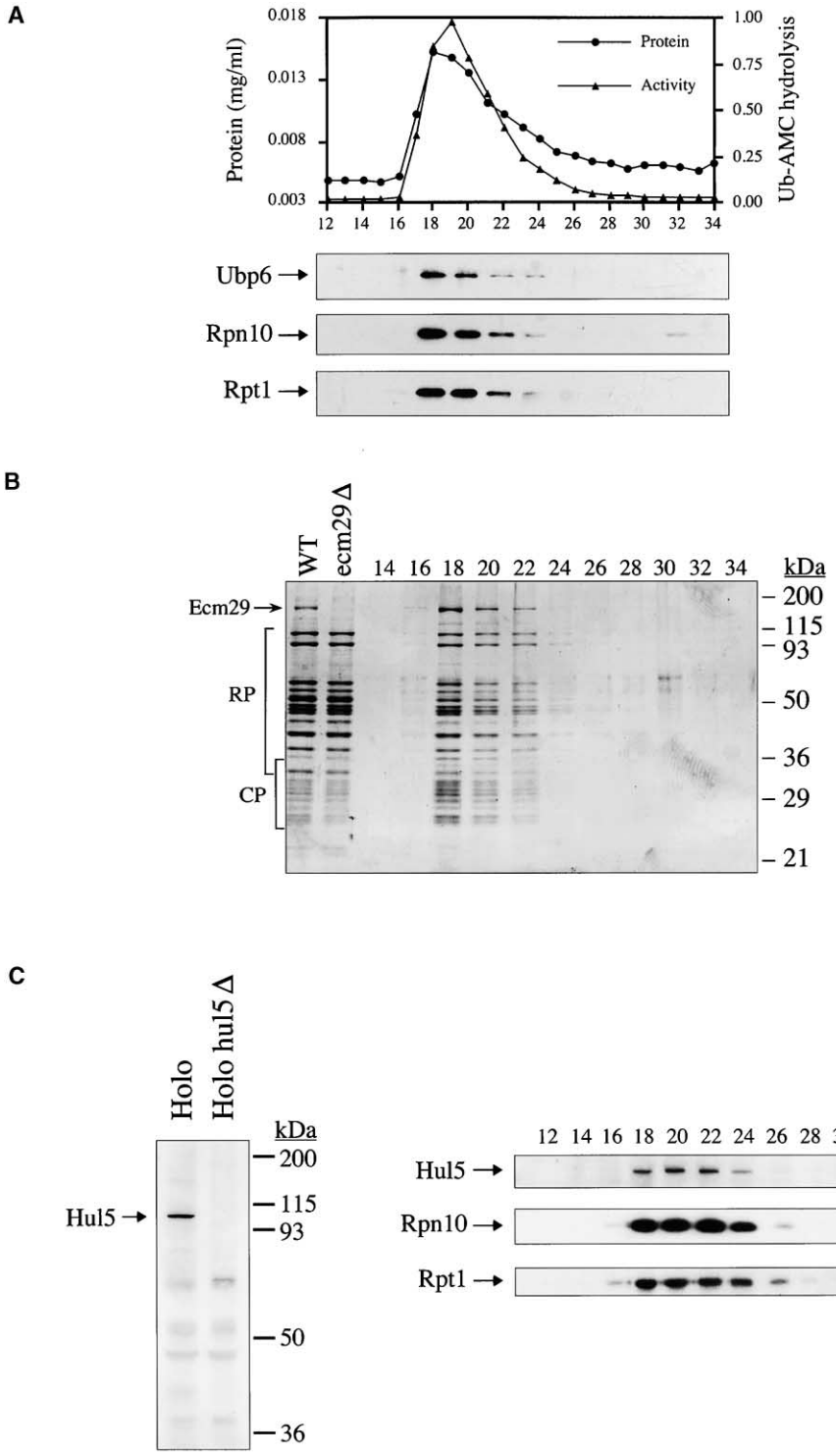

Figure 2. Gel Filtration Chromatography of Affinity-Purified Proteasomes

(A) Rpn11-TEV-ProA proteasomes were bound to IgG resin, washed with 100 mM NaCl, and released with TEV. The eluate was fractionated over a Superose 6 column in the absence of NaCl. Fractions were analyzed for protein content and LLVY-AMC-hydrolyzing activity and by SDS-PAGE/immunoblotting. Rpn10 and Rpt1 are subunits of the RP. LLVY-AMC-hydrolyzing activity was measured in arbitrary fluorescence units. The band assigned to Ubp6 is missing from *ubp6*Δ proteasomes (data not shown).

(B) Column fractions from the same experiment as in (A) were analyzed by 12.5% SDS-PAGE followed by Coomassie staining. Left-hand lanes, affinity-purified holoenzyme from wild-type and *ecm29*Δ yeast.

(C) Hul5 association with the proteasome. Left panel, affinity-purified proteasomes from wild-type and *hul5*Δ yeast were analyzed by 10% SDS-PAGE/immunoblotting, using antibodies to Hul5. Right panel, fractions from a Superose 6 column were analyzed by 10% SDS-PAGE/immunoblotting. The experiment was carried out as in (A), using anti-Hul5 antibody, except that the buffer used to wash the IgG column contained no NaCl.

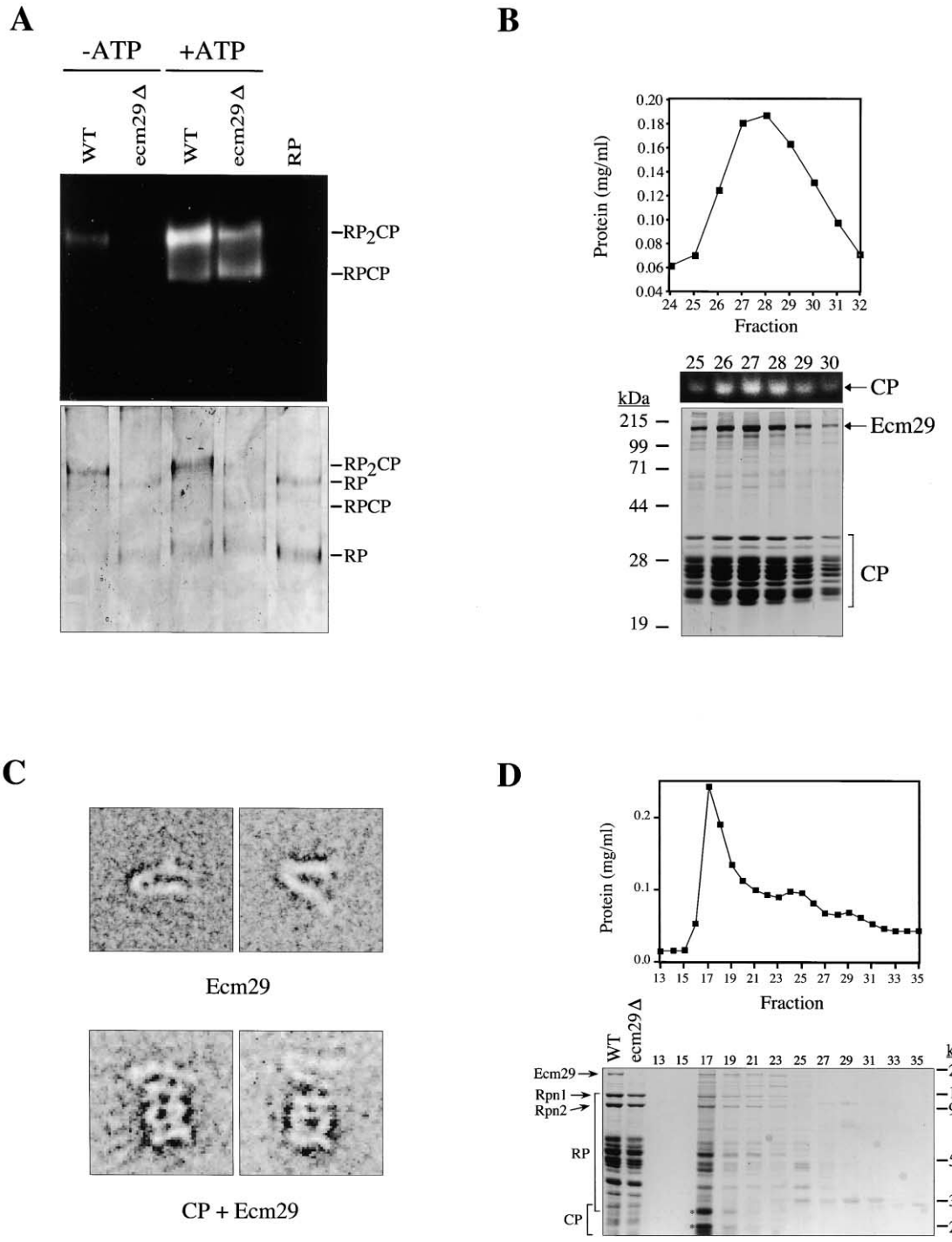

Figure 3. Ecm29 Tethers the CP to the RP

(A) Proteasomes were purified from an *RPN11-TEV-ProA* strain (WT) and an *ecm29*Δ *RPN11-TEV-ProA* strain in 100 mM NaCl and in the presence or absence of 1 mM ATP. 1 μg of each sample was analyzed by nondenaturing PAGE, and proteasomes were visualized by UV illumination after soaking the gels in LLVY-AMC (top). Purified RP was added as a marker (the slowly migrating RP band is presumably multimerized RP). Proteins were subsequently visualized with Coomassie (bottom).

(B) Complex formation between Ecm29 and the CP. Rpn11-TEV-ProA proteasomes were isolated using IgG resin and washed with 100 mM NaCl. Proteins step eluted using 300 mM NaCl were desalted, concentrated, and separated on a Superose 6 column in the absence of NaCl. Top, protein content; middle, CP activity assayed using LLVY-AMC after nondenaturing PAGE; bottom, 12.5% SDS-PAGE analysis followed by Coomassie staining.

(C) Electron micrographs of free Ecm29 and Ecm29-CP complexes. The Ecm29 and Ecm29-CP samples are from Superose-6 fractions 23 and 17, respectively (see Supplemental Data at http://molecule.org/cgi/content/full/10/3/495/DC1). The free Ecm29 images are averaged from 31 (left) and 29 (right) particles. Ecm29-CP images are from single particles. The side length of each panel is 40 nm.

(D) Complex formation between Ecm29 and the RP. Pre1-TEV-ProA proteasomes were processed as in (B). Top, protein content; bottom, 10% SDS-PAGE analysis followed by Coomassie staining. Bands marked by an asterisk are unidentified; however, there is essentially no CP in these fractions.

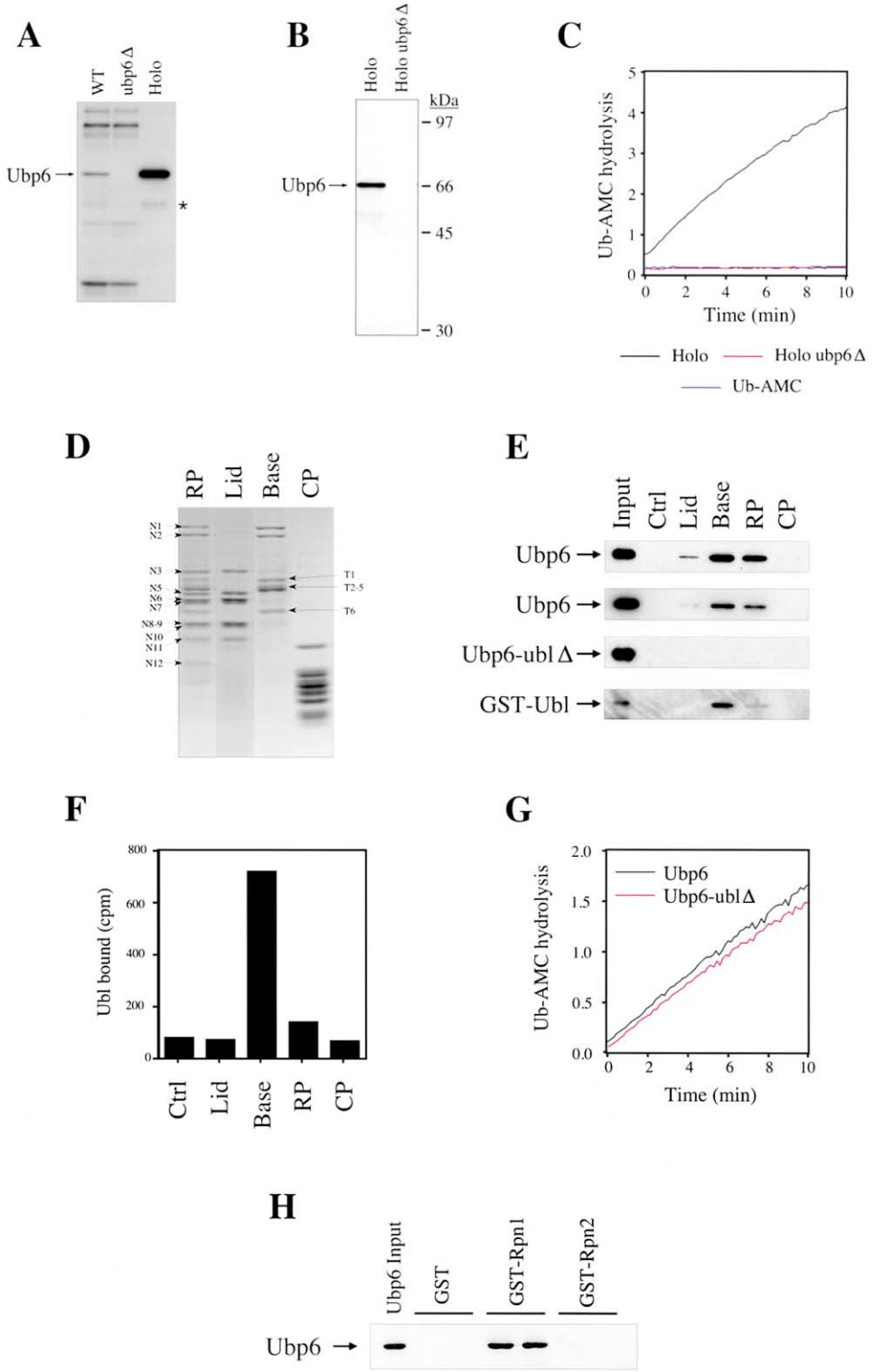

Figure 4. Ubp6 Associates with the RP and Is the Predominant Ubiquitin-AMC-Hydrolyzing Activity of the Yeast Proteasome

(A) [^{125}I]-UbVS treatment of wild-type whole-cell extract (40 μg), *ubp6*Δ extract (40 μg), and affinity-purified Rpn11-TEV-ProA holoenzyme (Holo; 2.5 μg). Proteins were resolved by 12.5% SDS-PAGE and visualized by autoradiography. A faint labeled product of lower molecular mass than Ubp6 (asterisk) is likely a breakdown product of Ubp6, since it is absent from both *ubp6*Δ whole-cell extracts (A) and *ubp6*Δ proteasomes (B).

(B) As (A), but with purified wild-type and *ubp6*Δ proteasomes (Rpn11 tagged). Proteasomes lacking Ubp6 have all the other proteasome components, including Ecm29, and show wild-type stability (data not shown).

(C) Ub-AMC hydrolysis assay using 2.5 nM purified wild-type or *ubp6*Δ proteasome. Hydrolytic rates were measured in arbitrary fluorescence units.

from *ubp6*Δ mutants (Figure 4B). To further evaluate the contribution of Ubp6 to proteasomal deubiquitinating activity, we used an independent assay involving ubiquitin-AMC (ubiquitin C-terminal 7-amido-4-methylcoumarin), a general substrate for deubiquitinating enzymes (Dang et al., 1998). Affinity-purified proteasomes contain abundant Ub-AMC-hydrolyzing activity (Figure 4C). In contrast, proteasomes purified from *ubp6*Δ mutants are virtually devoid of this activity (Figure 4C). These data confirm that Ubp6 is associated with the proteasome, and indicate that it represents the predominant Ub-AMC-hydrolyzing activity of the proteasome.

Ubp6 Associates with the Regulatory Particle via Its Ubiquitin-like Domain

To map the Ubp6 binding site in the proteasome, we used IgG resins loaded with purified lid, base, RP, and CP (Figure 4D), derived from strains expressing the appropriate tagged subunits (Figure 1A). Procedures for purifying these subcomplexes remove Ecm29, Hul5, and Ubp6 (Figure 4D; see Supplemental Data at http://molecule.org/cgi/content/full/10/3/495/DC1). The subcomplex-containing resins were incubated with bacterially expressed Ubp6, washed with buffer containing 100 mM NaCl, and then eluted with 500 mM NaCl. Ubp6 bound to the RP and to both of its subcomplexes, with the strength of binding following the order base $>$ RP $>$ lid (Figure 4E). Preferential binding of proteins to the base in comparison to the RP has been previously described (Braun et al., 1999) and might be a consequence of the complex structure of the RP (Walz et al., 1998), which could partially hinder access to ligand binding sites that are situated deep within the base. Ubp6 is composed of an N-terminal ubiquitin-like (Ubl) domain and a C-terminal catalytic domain (Wyndham et al., 1999). Since the ubiquitin-like proteins Rad23 and Dsk2 can be coimmunoprecipitated with the proteasome (Schauber et al., 1998; Wilkinson et al., 2001; Funakoshi et al., 2002), we tested whether the Ubl domain of Ubp6 was required for proteasome binding. Removal of the Ubl domain abolished proteasome binding (Figure 4E). The inability of Ubp6-ublΔ to bind proteasomes does not appear to reflect misfolding of the catalytic domain, because Ubp6 and Ubp6-ublΔ have similar Ub-AMC-hydrolyzing activity (Figure 4G) (see also Wyndham et al., 1999). The Ubl domain of Ubp6 was found to be sufficient for binding to the base, using both GST-Ubl and free radiolabeled Ubl domain (Figures 4E and 4F). In summary, the Ubl domain is necessary and sufficient for binding to the base, while binding to the lid is observed only when the catalytic domain of Ubp6 is present. We have recently found that Rpn1, a subunit of the base, is capable of binding the ubiquitin-like protein Rad23 (Elsasser et al., 2002). We therefore tested whether Rpn1 binds Ubp6 as well. Ubp6 was added to resins containing purified GST-Rpn1 or GST-Rpn2. Analysis of the glutathione (GSH) eluates indicated that Ubp6 does indeed bind Rpn1, suggesting that this may be the primary Ubp6 binding subunit of the base (Figure 4H). Coomassie staining of proteins in the eluates suggested that Rpn1 could, like the proteasome, bind approximately stoichiometric quantities of Ubp6 (data not shown).

Ubp6 Is Activated by Proteasome Binding

Recombinant Ubp6 was used to reconstitute the deubiquitinating activity found in affinity-purified proteasomes. Given that RP_2CP proteasomes are expected to contain two molecules of Rpn1, we used an approximately 4-fold molar excess of Ubp6 over proteasome (125-fold less Ubp6 than used in Figure 4G). Mixing of Ubp6 with *ubp6*Δ proteasomes resulted in a strong (~300-fold; see Supplemental Data at http://molecule.org/cgi/content/full/10/3/495/DC1) enhancement of Ubp6's Ub-AMC-hydrolyzing activity (Figure 5A). Recombinant Ubp6 restored the deubiquitinating activity of *ubp6*Δ proteasomes to an approximately wild-type level (Figure 5A). Higher Ubp6 levels did not result in appreciable further stimulation, presumably because specific Ubp6 binding sites had been saturated (data not shown). If physical association of Ubp6 with the proteasome is required for the stimulation of activity, then Ubp6-ublΔ should show no stimulation. Despite equivalent specific activities of purified Ubp6 and Ubp6-ublΔ (Figure 4G), only wild-type Ubp6 was stimulated by proteasomes (Figure 5A). The experiments described above suggested that binding of the Ubl domain of Ubp6 to the proteasome leads to activation of Ubp6. To test whether binding and activation are separable processes, we assayed proteasome subcomplexes for their ability to activate Ubp6. The RP stimulated Ubp6 efficiently, while base complexes stimulated Ub-AMC hydrolysis to an intermediate level (Figure 5B). Comparable results were obtained using the UbVS-mediated active site labeling assay for Ubp6 activation (Figure 5C). These results were surprising because the base is more profi-

(D) Composition of proteasome subcomplexes. Proteins from equal volumes of proteasome subcomplex resins were eluted by TEV protease, separated by 12.5% SDS-PAGE, and visualized by Coomassie. The purity of the subcomplex resins was further verified by SDS-PAGE at high protein loads (see Supplemental Data at http://molecule.org/cgi/content/full/10/3/495/DC1).

(E) Binding assays. Ubp6 derivatives were added in 2-fold molar excess to resins containing proteasome subcomplexes as shown in (D). Resins were washed with 100 mM NaCl and bound protein was then eluted with 500 mM NaCl. The same resins were used in parallel for all three proteins. Proteins in the eluates were separated by SDS-PAGE, transferred to nitrocellulose, and visualized using anti-Ubp6 or anti-GST (bottom) antibodies. The top two panels are different exposures. Similar results were obtained with endogenous Ubp6 (see Supplemental Data at http://molecule.org/cgi/content/full/10/3/495/DC1).

(F) ^{32}P-Ubp6-Ubl was added to resins containing proteasome subcomplexes. Resins were then washed as in (E), and eluates were subjected to scintillation counting.

(G) Ub-AMC-hydrolyzing activity of purified Ubp6 and Ubp6-ublΔ (0.5 μM). Activity was measured in arbitrary fluorescence units.

(H) Ubp6-Rpn1 complex formation. Recombinant Ubp6 was incubated with resin containing GST-fusion proteins as indicated. Resins were washed with 100 mM NaCl and eluted with glutathione. Eluted proteins were visualized using anti-Ubp6 antibodies after SDS-PAGE and immunoblotting. Paired lanes represent independent trials of the experiment. GST-Rpn1 and GST-Rpn2 were expressed at comparable levels.

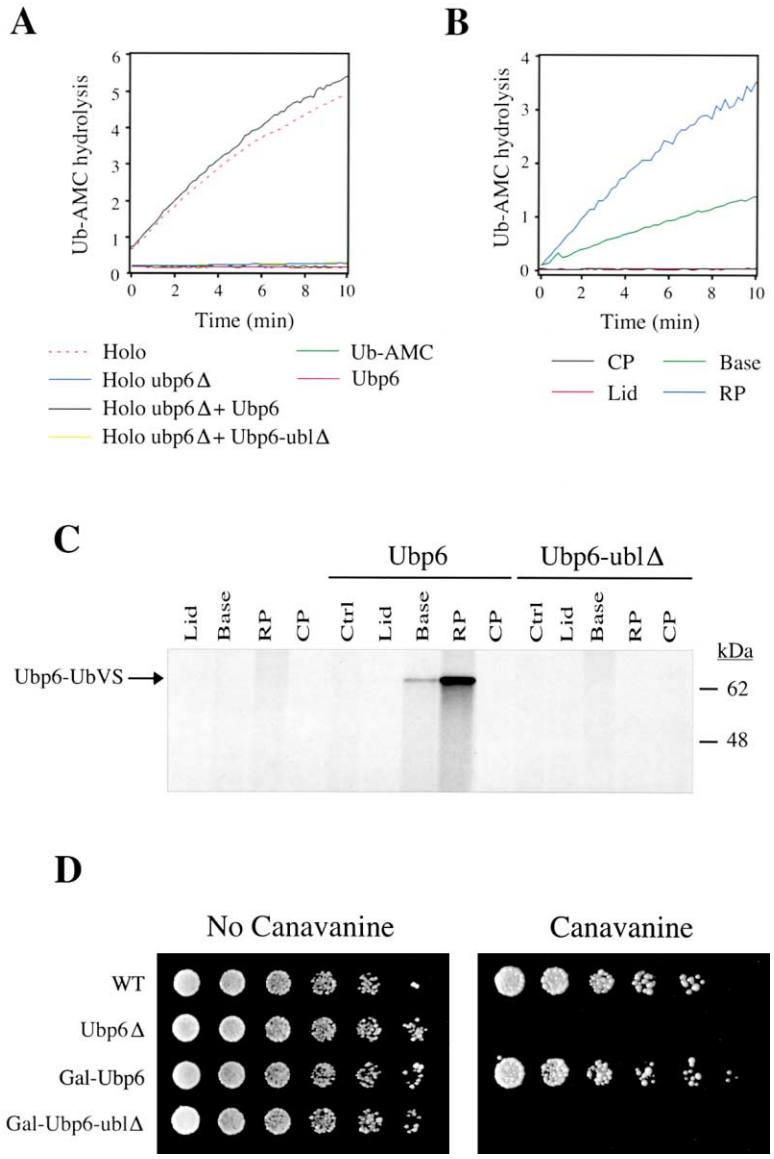

Figure 5. Ubp6 Is Activated by Association with the Proteasome

(A) Ub-AMC hydrolysis assays were performed on purified holoenzyme samples (1 nM), Ubp6 (4 nM), and reconstituted cocomplexes, as indicated. Ub-AMC, no enzyme control.

(B) Ub-AMC hydrolysis assays performed on proteasome subcomplexes (1 nM) in the presence of Ubp6 (4 nM). Complexes possessed near-baseline levels of Ub-AMC hydrolytic activity in the absence of added Ubp6.

(C) [^{125}I]-UbVS was reacted with equimolar amounts of proteasome subcomplexes and either Ubp6 or Ubp6-ublΔ. Samples were then analyzed by 10% SDS-PAGE and visualized by autoradiography. *Ctrl* samples contained [^{125}I]-UbVS and a Ubp6 derivative.

(D) 3-fold serial dilutions of wild-type (SUB62), *ubp6*Δ (YRB205), *GAL*-inducible *UBP6* (sDL147), and *GAL*-inducible *ubp6-ubl*Δ (sDL149) cells were spotted onto selective plates with or without 1.5 μg/ml canavanine sulfate and grown at 30°C for 3–5 days. *GAL* constructs were integrated at the *UBP6* locus.

cient than the RP in Ubp6 binding (Figure 4E). The greater efficiency of the RP in stimulating Ubp6 activity suggests a role for the lid in the activation mechanism. This model is further supported by the binding detected between the lid and full-length Ubp6 (Figure 4E).

In Vivo Requirement for the Ubp6-ubl Domain

Based on the requirement for the Ubl domain of Ubp6 in both proteasome binding and activation, *ubp6-ubl*Δ can be predicted to be a null mutation. Complete deletions of the *UBP6* gene result in sensitivity to canavanine (Figure 5D) (Amerik et al., 2000a). A comparable phenotype was observed when only the small (83 codon) *UBL* element was deleted (Figure 5D). Ubp6 and Ubp6-ublΔ were expressed at equivalent levels as determined by immunoblotting (data not shown). Given that the Ubl domain mediates activation of Ubp6 at the proteasome (Figure 5A), these data suggest that Ubp6 functions at the proteasome in vivo. Also yielding an apparent null phenotype was a Cys118 to Ala substitution of the active-site cysteine of Ubp6 (data not shown). This supports the view that the critical function of Ubp6 at the proteasome is indeed deubiquitination. As previously shown for *ubp6*Δ (Amerik et al., 2000a), the canavanine sensitivity of *ubp6-ubl*Δ strains was suppressed by overexpression of free ubiquitin, suggesting that canavanine toxicity is based on the depletion of free ubiquitin pools in this mutant (data not shown). *ubp6*Δ mutants are strongly defective in the degradation of Ub-Pro-β-galactosidase, a model substrate of the ubiquitin-proteasome pathway (Figure 6A). This phenotype could not be rescued by the catalytic domain (Ubp6-ublΔ; data not shown). Expression of the Ubl domain alone also failed to rescue this phenotype, while its overexpression stabilized Ub-Pro-β-gal in a genetic background that is wild-type for *UBP6* (data not shown). The latter finding is consistent with in vitro data indicating that the Ubl domain is sufficient for proteasome binding. A second model substrate, Ub-Leu-β-gal, was stabilized only modestly in the *ubp6*Δ mutant, indicating that Ubp6 is

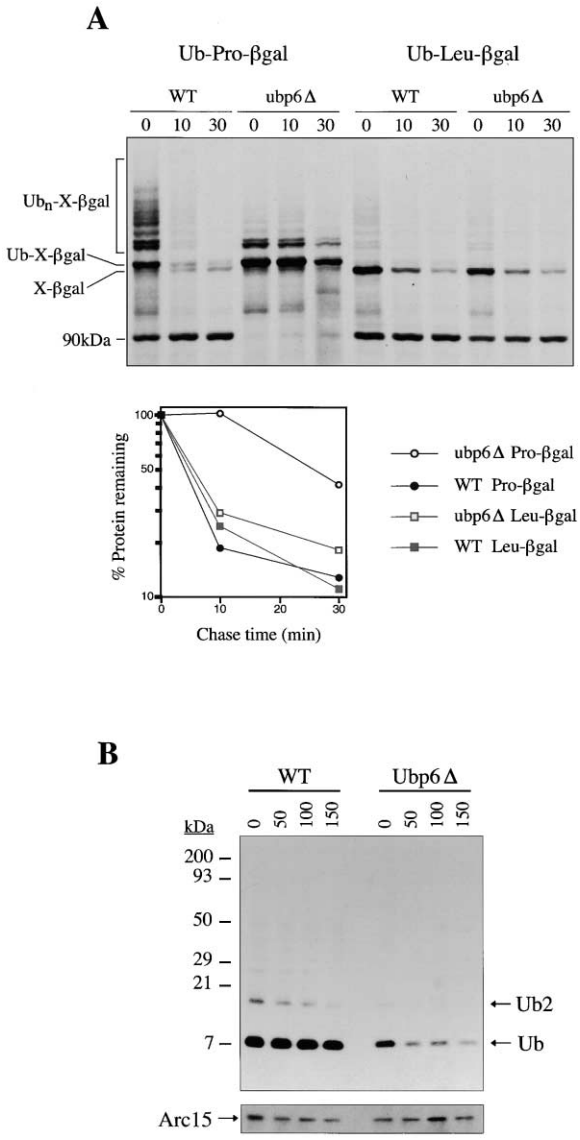

Figure 6. Phenotypic Characterization of *ubp6*Δ Mutants

(A) Stabilization of Ub-Pro-β-gal in *ubp6*Δ mutants (YRB202). Turnover rates for Ub-Pro-β-gal and Ub-Leu-β-gal proteins were assessed by [^{35}S]-pulse-chase analysis. Chase incubations were carried out for 0–30 min, as indicated. Immunoprecipitations were carried out using anti-β-gal antibodies, followed by SDS-PAGE and fluorography. The 90 kDa band is a partially degraded form of β-gal, whose levels typically correlate with degradation rates of the full-length protein. β-gal turnover was quantified as described (Baker and Varshavsky, 1991).

(B) Ubiquitin turnover was assessed in wild-type (SUB62) and *ubp6*Δ (YRB205) cells during logarithmic growth. Cycloheximide was added, and aliquots, normalized by optical density, were taken at the indicated times. Proteins were subjected to 20% SDS-PAGE/immunoblot analysis using anti-ubiquitin antibodies. Ub_2, ubiquitin dimer. An antibody to Arc15 was used to control for protein load.

not required for all proteasome-mediated protein degradation (Figure 6A).

Ubiquitin Is Unstable in *ubp6*Δ Mutants

One possible function for a proteasome-associated deubiquitinating enzyme is to facilitate substrate translocation into the CP by removing ubiquitin from conjugates. A defect in this process should retard degradation of the substrate, assuming that attached ubiquitin hinders translocation. However, this model predicts an accumulation of high molecular weight ubiquitin-protein conjugates in *ubp6*Δ mutants, which has not been observed (Figure 6) (Amerik et al., 2000a; data not shown). Alternatively, the primary function of Ubp6 could be to ensure the regeneration of ubiquitin at the proteasome. In this case, the *ubp6*Δ mutation would convert ubiquitin into a metabolically unstable protein. The stability of ubiquitin in *ubp6*Δ mutants was tested after treatment with cycloheximide to inhibit the synthesis of new ubiquitin. Consistent with the model that Ubp6 removes ubiquitin from conjugates bound to the RP, ubiquitin was found to be unstable in these mutants (Figure 6B). Given that Ubp6 activity is localized to the proteasome, the instability of ubiquitin in *ubp6*Δ mutants appears to reflect an alteration of the fate of conjugated ubiquitin bound to the proteasome.

Discussion

Composition of the Proteasome

Since its identification, the proteasome holoenzyme has been studied in a form whose subunit composition in vitro is closely similar across the eukaryotic kingdom. The present study defines a different form of the proteasome, which we suggest to be a closer approximation of the particle as it exists in vivo. As previously defined, proteasomes lack important components as a result of exposure to nonphysiological salt concentrations during purification and are therefore unstable in vitro and deficient in deubiquitinating activity. The major components that differentiate conventional and affinity-purified proteasomes, Ecm29 and Ubp6, explain these functional differences. The present findings should allow improved in vitro analysis of this particle and are likely to be relevant to mammalian proteasomes as well, since the three components that we have identified—Ecm29, Hul5, and Ubp6—are evolutionarily conserved. Indeed, homologs of Hul5 and Ubp6 are thought to interact with mammalian proteasomes, although it is not known whether they are abundant or functionally significant components of the mammalian complex (You and Pickart, 2001; Borodovsky et al., 2001; see also Verma et al., 2000 regarding yeast Ubp6). The phenotype of *hul5* mutants has yet to be characterized. Interestingly, the mammalian homolog of Hul5, KIAA10, may bind proteasomes through the S2/Rpn1 subunit (You and Pickart, 2001), as appears to be the case for Rad23 (Elsasser et al., 2002) and Ubp6. This raises the remarkable possibility that Rpn1 is used to couple proteasomes to ubiquitin binding (Rad23), deubiquitinating (Ubp6), and ubiquitin-protein-ligating (Hul5) activities.

The CP-RP association within proteasomes is stable in vivo; though they are prone to dissociate in vitro (Hendil et al., 2002). Our findings suggest that Ecm29 provides for stable complex formation as observed within cells. Electron microscopic studies indicate that the RP and CP contact one another directly only near the center of their interface (Walz et al., 1998). The connection is loose and allows for considerable motion of the RP with

Table 1. Yeast Strains

Strain	Genotype
SUB62	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1*
SDL66	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpn11::RPN11-TEVProA (HIS3)*
SDL73	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpn11::RPN11-TEVProA (HIS3)*
SDL135	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 pre1::PRE1-TEVProA (HIS3)*
SY36	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt1::HIS3 pEL36 (TRP1)*
SDL145	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 ubp6::URA3 rpn11::RPN11-TEVProA (HIS3)*
YRB205	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 ubp6::URA3*
YRB202	*MATa leu2-3, 2-112, ura3-52, his4, ade1, ubp6::URA3*
SDL147	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 ubp6::GAL-Ubp6 (HIS3)*
SDL149	*MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 ubp6::GAL-Ubp6-ublΔ (HIS3)*
10933[a]	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ecm29::kanMX4*
BY4741[a]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
SDL91	*MATα his3 leu2 ura3 rpn11::RPN11-TEVProA (HIS3) ecm29::kanMX4*
SDL89	*MATa his3 leu2 ura3 lys2 rpn11::RPN11-TEVProA (HIS3)*

SDL91 and SDL89 were created by mating SDL66 with 10933 and SDL73 with BY4741, respectively.
[a] Obtained from Research Genetics (Brachmann et al., 1998)

respect to the CP. Ecm29 appears to bind the CP near the interface region, consistent with its function in stabilizing CP-RP association.

Deubiquitination and Proteasome Function

Of special interest is the proteasomal association of Ubp6 and its implications for the coupling of deubiquitination and protein degradation. According to previous work, several additional deubiquitinating enzymes may be localized to the proteasome, and distinct functions have been proposed for these enzymes. For example, deubiquitinating enzymes could function in the proteasome as editing activities (Lam et al., 1997), progressively shortening proteasome-bound ubiquitin chains from their distal end, so as to limit the time of proteasome occupancy for any single conjugate. Loss of function in an editing isopeptidase should lead to enhanced degradation of at least some ubiquitinated proteins, which has not been observed in *ubp6*Δ mutants. An editing function appears to be mediated by Uch37, which is an integral subunit of proteasomes from mammals (Lam et al., 1997), with probable orthologs in *D. melanogaster* (Holzl et al., 2000) and *S. pombe* (Li et al., 2000). However, a significant role for Uch37 in ubiquitin regeneration has not been suggested. Moreover, no homolog of Uch37 appears to be present in proteasomes from *S. cerevisiae* (Glickman et al., 1998a; this work), and no discernable homolog is encoded in the *S. cerevisiae* genome. The Doa4 deubiquitinating enzyme has been suggested to play a critical role in ubiquitin regeneration at the proteasome (Papa and Hochstrasser, 1999). Doa4 can regenerate ubiquitin from membrane proteins targeted by ubiquitination to the vacuole, a process in which the proteasome has not been implicated (Amerik et al., 2000b; Katzmann et al., 2001). Like *ubp6*Δ, mutants in *DOA4* exhibit enhanced ubiquitin turnover. However, this *doa4* phenotype is suppressed in vacuolar protease-deficient mutants (Swaminathan et al., 1999), indicating that ubiquitin is degraded within the vacuole in these strains. We suggest that Ubp6 and Doa4 function in parallel to recover ubiquitin from conjugated species involved in distinct proteasomal and vacuolar pathways, respectively.

Although several deubiquitinating enzymes are thought to be associated with the proteasome, our data suggest that Ubp6 is of particular importance. Like Uch37, it is abundant within proteasomes, but only Ubp6 is general to eukaryotes. In addition, *ubp6*Δ mutants are canavanine sensitive and strongly defective in the degradation of Ub-Pro-β-gal and thus appear to have a stronger phenotype than do mutants in the *S. pombe* homolog of Uch37 (Li et al., 2000). Although the exact division of labor among the various deubiquitinating enzymes associated with the proteasome remains to be determined, the multiplicity of such enzymes is itself surprising. The functional relationships among them should be clarified by comparative studies of their phenotypes, substrate specificities, and location within the RP. Current data suggest a distinction between "editing" deubiquitinating enzymes and those that spare ubiquitin from degradation by the CP. However, the mechanistic basis for this distinction is not clear. Because ubiquitin-protein conjugates are targeted to the proteasome by virtue of their ubiquitin chains, premature deubiquitination may interfere with substrate degradation by dissociating the substrate from the proteasome. Presumably there are safeguards on the activity of enzymes such as Ubp6 to prevent interference with substrate degradation, perhaps allowing them to act only on substrates that are committed to degradation.

Localized Activation of Ubp6

Ubp6 is dramatically activated by proteasome binding, implying that it functions obligatorily as a proteasome component. Activation of Ubp6 is unlikely to be peculiar to yeast since, in mammalian cell extracts, only proteasome-bound Usp14 (the Ubp6 homolog) is active and can be labeled by UbVS (A.B. and H.P., unpublished data). In mammals, UbVS labeling of proteasomal Usp14 can be further stimulated, though less dramatically, by proteasome inhibition (Borodovsky et al., 2001). Although the relationship between this activation effect and that described in the present work is unclear, it is possible that Usp14 is subject to multiple controls within the proteasome.

One implication of localized activation is that ubiquitin

turnover in *ubp6*Δ mutants should reflect a failure to regenerate ubiquitin within the proteasome. Possible reactions involved in ubiquitin regeneration include (A) progressive removal of ubiquitin groups from the distal end of chains, (B) *en bloc* removal of intact chains from the chain-substrate junction, and (C) disassembly of free chains produced through the reaction B. Given the potential complexity of this process, it is possible that other deubiquitinating enzymes can cooperate with Ubp6 at the proteasome. Such isopeptidases could be either integral subunits of the holoenzyme that possess deubiquitinating activity but have escaped detection due to a restricted substrate specificity, or soluble deubiquitinating enzymes. However, soluble deubiquitinating enzymes may have hindered access to the interior of the RP, particularly while it is occupied by a ubiquitin-protein conjugate.

An additional question raised by this study is the mechanism of Ubp6 activation by the proteasome. Deubiquitinating enzymes fall into two families, UCHs (ubiquitin C-terminal hydrolases) and UBPs (ubiquitin-specific proteases), with Ubp6 among the latter. Some UCHs are thought to be regulated by a ~20 residue peptide segment that occludes the active site in the basal state of the enzyme but can be displaced upon binding of proper substrates (Johnston et al., 1999). Whether such "active-site crossover loops" occur in UBPs is not known. We suggest an alternative mechanism, namely that Ubp6 is activated by the proteasome's provision of part or all of a ubiquitin binding site that positions ubiquitin within the active site of Ubp6. In support of this possibility, free Usp14, the mammalian homolog of Ubp6, has an unusually high K_M for ubiquitin (>50 μM) (Yin et al., 2000). Our finding that the lid is required for full activation is interesting in light of the role of the COP9 signalosome in disassembly of conjugates between the ubiquitin-like protein Rub1/Nedd-8 and its target proteins (Lyapina et al., 2001). Given the evolutionary kinship between the COP9 signalosome and the proteasome lid (Glickman et al., 1998b), these data suggest that at least one biochemical function, conjugate disassembly, may be similarly regulated by the two protein complexes.

Deubiquitination by Ubp6 Rescues Ubiquitin from Degradation

The metabolic stability of ubiquitin in wild-type yeast and mammalian cells (Haas and Bright, 1987; Swaminathan et al., 1999) suggests that ubiquitin chains which target attached proteins to the proteasome are not degraded together with substrate. If not degraded, the chains are presumably released from the substrate by one or more deubiquitinating enzymes prior to substrate translocation into the CP. Our results indicate that the ability of proteasomes to degrade substrates while regenerating ubiquitin is mediated at least in part by Ubp6. The binding of Ubp6 to the base, and to Rpn1 in particular, suggests that Ubp6 may be positioned deep within the RP, close to the pathway of substrate translocation to the CP. The base of the RP contacts the CP and is thought to mediate ubiquitin-chain binding (Lam et al., 2002) as well as unfolding of the target protein (Braun et al., 1999). It is highly unlikely that the CP channel could accommodate ubiquitin unless it was first unfolded. Ubiquitin can thus presumably be unfolded by the proteasome and translocated into the CP like its conjugative target protein. This is surprising because ubiquitin has an unusually stable tertiary structure; it is intact at 85°C as well as pH extremes of 1 and 13 (Lenkinski et al., 1977). The structural stability of ubiquitin may underlie its dramatic sequence conservation among eukaryotes. That is, ubiquitin may have evolved to be at least partially refractory to the unfoldase activity of the proteasome. The structural stability of ubiquitin is evidently not sufficient to prevent its translocation into the core particle and subsequent degradation. Most likely, resistance of ubiquitin to unfolding and spatially restricted, Ubp6-dependent deubiquitination events both contribute to uncoupling of the metabolic fates of ubiquitin and its conjugative targets.

Experimental Procedures

Yeast Strains

See Table 1 for yeast strains. SUB62 was used as an isogenic control unless otherwise stated. SDL89 was used as a control for sDL91. Plasmids and ProA-tagging methods are described in the Supplemental Data at http://molecule.org/cgi/content/full/10/3/495/DC1.

Affinity Purification of Proteasomes and Proteasome Subcomplexes

Proteasome holoenzyme can be purified using any of the ProA-tagged strains, but Rpn11-TEV-ProA was routinely used for this. RP and lid were prepared using Rpn11-TEV-ProA, base using ProA-TEV-Rpt1, and CP using Pre1-TEV-ProA. The proteasome and its subcomplexes were purified in the presence or absence of 1 mM ATP as indicated. Cells were harvested, resuspended in a 2-fold volume of buffer 1 (50 mM Tris-HCl [pH 8], 1 mM EDTA), and lysed by French press. Lysate was clarified at 15,000 × g for 25 min, incubated with IgG resin (ICN) for 1 hr at 4°C, and the resin washed with 50 bed volumes buffer 2 (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100 mM NaCl). At this stage, the proteasome is essentially pure electrophoretically and may be eluted using TEV protease or used to produce proteasome subcomplexes as described below. Holoenzyme was generated by washing the IgG resin with 3 vol TEV elution buffer (TEB; 50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM DTT), then incubating with 1.5 vol TEB containing 150 U of 6His-TEV protease at 30°C for 1 hr. Holoenzyme was eluted with TEB, and the TEV protease was removed by incubation with Nickel-NTA resin (Qiagen) at 4°C for 15 min. Proteasomes were stored at −80°C in 10% glycerol. From 10 liter of early stationary culture, a yield of 3 mg is typical.

RP and CP were generated by incubating IgG resin with buffer 3 (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 500 mM NaCl) for 1 hr at 4°C. Resin was washed with 50 vol buffer 3 followed by 5 vol buffer 2. Alternatively, RP and CP were generated by washing resin with 50 vol buffer 4 (50 mM Tris-HCl [pH7.5], 1 mM EDTA, 300 mM NaCl) followed by 5 vol buffer 2. RP or CP were then eluted from the column by TEV protease as described above and stored at −80°C in 10% glycerol. Base and lid were generated by incubating the IgG resin with buffer 5 (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 M NaCl) for 1 hr at 23°C. The resin was washed with 50 vol buffer 5, then 5 vol buffer 2. Base or lid was eluted by TEV protease.

Active Site Labeling of Ubp6

Recombinant purified Ubp6 or Ubp6-ublΔ (0.67 pmol) was mixed with equimolar amounts of proteasome subcomplexes as indicated. Samples were preincubated in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 100 mM NaCl, and 0.1 mg/ml ovalbumin for 2 hr at 30°C. ^{125}I-UbVS (10^6 cpm), prepared as described (Borodovsky et al., 2001), was added, and labeling was stopped after 1 hr by addition of SDS-PAGE loading buffer.

Ubp6 Binding Experiments

Recombinant Ubp6 (180 μg), Ubp6-ublΔ (180 μg) or GST-Ubl (360 μg) were incubated with subcomplex resins (above) in buffer 9 (50 mM Tris-HCl [pH 7.5], 1 mM EDTA) for 15 min at 4°C. Columns were washed with 50 vol buffer 2 and then eluted with buffer 3. Equivalent volumes of eluate were analyzed by SDS-PAGE. Alternatively, ^{32}P-Ubl was added to subcomplex resins, and column eluates were analyzed by liquid scintillation counting. To assay Ubp6 binding to Rpn1, BL21 cells expressing GST, GST-Rpn1, and GST-Rpn2 were lysed by French press in 25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM DTT, and 0.5 mM EDTA, and the lysate was clarified at 25,000 × g for 20 min. BSA was added to the lysates (0.1 mg/ml), and lysates were incubated with glutathione resin for 1 hr at 4°C. The resin was washed 3× with 4 vol/wash of lysis buffer containing BSA. Recombinant Ubp6 (20 μg) was incubated with each column for 40 min at 4°C. Resins were washed 3× with 4 vol/wash of lysis buffer, then eluted with lysis buffer containing 30 mM glutathione.

Assays of Proteasome Activity

Solution and gel-based assays of proteasome activity were carried out as described (Glickman et al., 1998a).

Additional methods are described in Supplemental Data at http://molecule.org/cgi/content/full/10/3/495/DC1.

Acknowledgments

We are especially grateful to B. Petre for technical assistance, and to C. Enenkel, who helped with ProA-tagging. We also thank C. Mann and R. Vierstra for generously providing antibodies, and S. Elsasser for providing the ProA-TEV-Rpt1 strain. This work was supported by NIH grant GM43601 (D.F.), by NIH grants to H.P., and by fellowships from the NIH (D.S.L.) and Deutsche Forschungsgemeinschaft (M.S.). J.H. was supported by the MSTP Program through grant T32 GM07753 to Harvard Medical School. The molecular electron microscopy facility at Harvard Medical School was established by a donation from the Giovanni Armenise Harvard Center for Structural Biology and maintained through NIH grant GM62580.

Received: May 20, 2002
Revised: July 3, 2002

References

Amerik, A.Y., Li, S.-J., and Hochstrasser, M. (2000a). Analysis of the deubiquitinating enzymes of the yeast *Saccharomyces cerevisiae*. Biol. Chem. *381*, 981–992.

Amerik, A.Y., Nowak, J., Swaminathan, S., and Hochstrasser, M. (2000b). The Doa4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways. Mol. Biol. Cell *11*, 3365–3380.

Baker, R.T., and Varshavsky, A. (1991). Inhibition of the N-end rule pathway in living cells. Proc. Natl. Acad. Sci. USA *88*, 1090–1094.

Borodovsky, A., Kessler, B.M., Casagrande, R., Overkleeft, H.S., Wilkinson, K.D., and Ploegh, H.L. (2001). A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14. EMBO J. *20*, 5187–5196.

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., and Boeke, J.D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast *14*, 115–132.

Braun, B.C., Kloetzel, P.-M., Kraft, R., Dahlmann, B., Glickman, M.H., Finley, D., and Schmidt, M. (1999). The base of the proteasome regulatory complex exhibits ATP-dependent chaperone-like activity. Nat. Cell Biol. *1*, 221–226.

Cagney, G., Uetz, P., and Fields, S. (2001). Two-hybrid analysis of the *Saccharomyces cerevisiae* 26S proteasome. Physiol. Genomics *7*, 27–34.

Dang, L.C., Melandri, F., and Stein, R.L. (1998). Kinetic and mechanistic studies on the hydrolysis of ubiquitin C-terminal 7-amido-4-methylcoumarin by deubiquitinating enzymes. Biochemistry *37*, 1868–1879.

Davy, A., Bello, P., Thierry-Mieg, N., Vaglio, P., Hitti, J., Doucette-Stamm, L., Thierry-Mieg, D., Reboul, J., Boulton, S., Walhout, A.J., et al. (2001). A protein-protein interaction map of the *Caenorhabditis elegans* 26S proteasome. EMBO Rep. *2*, 821–828.

Elsasser, S., Gali, R.R., Schwickart, M., Larsen, C.N., Leggett, D.S., Müller, B., Feng, M.T., Tübing, F., Dittmar, G.A.G., and Finley, D. (2002). Proteasome subunit Rpn1 binds ubiquitin-like protein domains. Nat. Cell Biol. *4*, 725–730.

Finley, D. (2002). Ubiquitin chained and crosslinked. Nat. Cell Biol. *4*, E121–E123.

Funakoshi, M., Sasaki, T., Nishimoto, T., and Kobayashi, H. (2002). Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome. Proc. Natl. Acad. Sci. USA *99*, 745–750.

Gavin, A.C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J.M., Michon, A.M., Cruciat, C.M., et al. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature *415*, 141–147.

Glickman, M.H., Rubin, D.M., Fried, V.A., and Finley, D. (1998a). The regulatory particle of the *Saccharomyces cerevisiae* proteasome. Mol. Cell. Biol. *18*, 3149–3162.

Glickman, M.H., Rubin, D.M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V.A., and Finley, D. (1998b). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell *94*, 615–623.

Groll, M., Ditzel, L., Löwe, J., Stock, D., Bochtler, M., Bartunik, H.D., and Huber, R. (1997). Structure of 20S proteasome from yeast at 2.4 Å resolution. Nature *386*, 463–471.

Groll, M., Bajorek, M., Köhler, A., Moroder, L., Rubin, D.M., Huber, R., Glickman, M.H., and Finley, D. (2000). A gated channel into the proteasome core particle. Nat. Struct. Biol. *7*, 1062–1067.

Haas, A.L., and Bright, P.M. (1987). The dynamics of ubiquitin pools within cultured human lung fibroblasts. J. Biol. Chem. *262*, 345–351.

Hendil, K.B., Hartmann-Peterson, R., and Tanaka, K. (2002). 26S proteasomes function as stable entities. J. Mol. Biol. *315*, 627–636.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Ho, Y., Gruhler, A., Heilbut, A., Bader, G.D., Moore, L., Adams, S.L., Millar, A., Taylor, P., Bennett, K., Boutilier, K., et al. (2002). Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. Nature *415*, 180–183.

Holzl, H., Kapelari, B., Kellerman, J., Seemuller, E., Sumegi, M., Udvardy, A., Medalia, O., Sperling, J., Muller, S.A., Engel, A., et al. (2000). The regulatory complex of *Drosophila melanogaster* 26S proteasomes. J. Cell Biol. *150*, 119–130.

Jager, S., Strayle, J., Heinemeyer, W., and Wolf, D.H. (2001). Cic1, an adaptor protein specifically linking the 26S proteasome to its substrate. EMBO J. *20*, 4423–4431.

Johnston, S.C., Riddle, S.M., Cohen, R.E., and Hill, C.P. (1999). Structural basis for the specificity of ubiquitin C-terminal hydrolases. EMBO J. *18*, 3877–3887.

Kaiser, P., Moncollin, V., Clarke, D.J., Watson, M.H., Bertolaet, B.L., Reed, S.I., and Bailly, E. (1999). Cyclin-dependent kinase and Cks/Suc1 interact with the proteasome in yeast to control proteolysis of M-phase targets. Genes Dev. *13*, 1190–1202.

Katzmann, D.J., Babst, M., and Emr, S.D. (2001). Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell *106*, 145–155.

Köhler, A., Cascio, P., Leggett, D.S., Woo, K.-M., Goldberg, A.L., and Finley, D. (2001). The axial channel of the proteasome core particle is gated by the Rpt2 ATPase and controls both substrate entry and product release. Mol. Cell *7*, 1143–1152.

Lam, Y.A., Xu, W., DeMartino, G.N., and Cohen, R.E. (1997). Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. Nature *385*, 737–740.

Lam, Y.A., Lawson, T.G., Velayutham, M., Zweier, J.L., and Pickart,

C.M. (2002). A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. Nature *416*, 763–767.

Lenkinski, R.E., Chen, D.M., Glickson, J.D., and Goldstein, G. (1977). Nuclear magnetic resonance studies on the denaturation of ubiquitin. Biochim. Biophys. Acta *494*, 126–130.

Li, T., Navi, N.I., Yang, H., and Teo, T.S. (2000). Identification of a 26S proteasome-associated UCH in fission yeast. Biochem. Biophys. Res. Commun. *272*, 270–275.

Lussier, M., White, A.M., Sheraton, J., di Paolo, T., Treadwell, J., Southard, S.B., Horenstein, C.I., Chen-Weiner, J., Ram, A.F., Kapteyn, J.C., et al. (1997). Large-scale identification of genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. Genetics *147*, 435–450.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., Shevchenko, A., and Deshaies, R.J. (2001). Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. Science *292*, 1382–1385.

Papa, F., and Hochstrasser, M. (1999). Interaction of the Doa4 deubiquitinating enzyme with the yeast 26S proteasome. Mol. Biol. Cell *10*, 741–756.

Park, K.C., Woo, S.K., Yoo, Y.J., Wyndham, A.M., Baker, R.T., and Chung, C.H. (1997). Purification and characterization of Ubp6, a new ubiquitin-specific protease in *Saccharomyces cerevisiae*. Arch. Biochem. Biophys. *347*, 78–84.

Schauber, C., Chen, L., Tongaonakar, P., Vega, I., Lambertson, D., Potts, W., and Madura, K. (1998). Rad23 links DNA repair to the ubiquitin/proteasome pathway. Nature *391*, 715–718.

Swaminathan, S., Amerik, A.Y., and Hochstrasser, M. (1999). The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast. Mol. Biol. Cell *10*, 2583–2594.

Tongaonkar, P., Chen, L., Lambertson, D., Ko, B., and Madura, K. (2000). Evidence for an interaction between ubiquitin-conjugating enzymes and the 26S proteasome. Mol. Cell. Biol. *20*, 4691–4698.

Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R.J. (2000). Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Mol. Biol. Cell *11*, 3425–3439.

Verma, R., McDonald, H., Yates, J.R., III, and Deshaies, R.J. (2001). Selective degradation of ubiquitinated Sic1 by purified 26S proteasome yields active S phase cyclin-cdk. Mol. Cell *8*, 439–448.

Walz, J., Erdmann, A., Kania, M., Typke, D., Koster, A.J., and Baumeister, W. (1998). 26S proteasome structure revealed by three-dimensional electron microscopy. J. Struct. Biol. *121*, 19–29.

Wang, G., Yang, J., and Huibregtse, J.M. (1999). Functional domains of the Rsp5 ubiquitin-protein ligase. Mol. Cell. Biol. *19*, 342–352.

Wei, N., Tsuge, T., Serino, G., Dohmae, N., Takio, K., Matsui, M., and Deng, X.W. (1998). The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex. Curr. Biol. *8*, 919–922.

Whitby, F.G., Masters, E.I., Kramer, L., Knowlton, J.R., Yao, Y., Wang, C.C., and Hill, C.P. (2000). Structural basis for the activation of 20S proteasomes by 11S regulators. Nature *408*, 115–120.

Wilkinson, C.R.M., Seeger, M., Hartmann-Peterson, R., Stone, M., Wallace, M., Semple, C., and Gordon, C. (2001). Proteins containing UBA domains are able to bind multiubiquitin chains. Nat. Cell Biol. *3*, 939–943.

Wyndham, A.M., Baker, R.T., and Chelvanayagam, G. (1999). The Ubp6 family of deubiquitinating enzymes contains a ubiquitin-like domain. Protein Sci. *8*, 1268–1275.

Xie, Y., and Varshavsky, A. (2000). Physical association of ubiquitin ligases and the 26S proteasome. Proc. Natl. Acad. Sci. USA *97*, 2497–2502.

Yin, L., Krantz, B., Russell, N.S., Deshpande, S., and Wilkinson, K.D. (2000). Nonhydrolyzable diubiquitin analogues are inhibitors of ubiquitin conjugation and deconjugation. Biochemistry *39*, 10001–10010.

You, J., and Pickart, C.M. (2001). A HECT domain E3 enzyme assembles novel polyubiquitin chains. J. Biol. Chem. *276*, 19871–19878.