Complementary Roles for Rpn11 and Ubp6 in Deubiquitination and Proteolysis by the Proteasome

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Substrates destined for degradation by the 26 S proteasome are labeled with polyubiquitin chains. These chains can be dismantled by deubiquitinating enzymes (DUBs). A number of reports have identified different DUBs that can hydrolyze ubiquitin from substrates bound to the proteasome. We measured deubiquitination by both isolated lid and base-core particle subcomplexes, suggesting that at least two different DUBs are intrinsic components of 26 S proteasome holoenzymes. In agreement, we find that highly purified proteasomes contain both Rpn11 and Ubp6, situated within the lid and base subcomplexes, respectively. To study their relative contributions, we purified proteasomes from a mutant in the putative metalloprotease domain of Rpn11 and from a ubp6 null. Interestingly, in both preparations we observed slower deubiquitination rates, suggesting that Rpn11 and Ubp6 serve complementary roles. In accord, the double mutant is synthetically lethal. In contrast to WT proteasomes, proteasomes lacking the lid subcomplex or those purified from the rpn11 mutant are less sensitive to metal chelators, supporting the prediction that Rpn11 may be a metalloprotein. Treatment of proteasomes with ubiquitin-aldehyde or with cysteine modifiers also inhibited deubiquitination but simultaneously promoted degradation of a monoubiquitinated substrate along with the ubiquitin tag. Degradation is unique to 26 S proteasome holoenzymes; we could not detect degradation of a ubiquitinated protein by “lidless” proteasomes, although they were competent for deubiquitination. The fascinating observation that a single ubiquitin moiety is sufficient for targeting an otherwise stable substrate to proteasomes exposes how rapid deubiquitination of poorly ubiquitinated substrates may counteract degradation.

The 26 S proteasome is the protease responsible for most regulatory intracellular proteolysis in eukaryotes (1–4). Elimination of proteins by the proteasome usually requires covalent attachment of the carboxyl terminus of polyubiquitin chains to an amino group on the substrate (5–7). This process is reversible by deubiquitinating enzymes (DUBs); reviewed in Refs. 8–11. The 26 S proteasome can be divided into two subcomplexes, the 670-kDa 20 S proteolytic core particle (CP) where proteolysis occurs, and the 900-kDa 19 S regulatory particle (RP), which in turn is composed of a lid and a base (12). Attachment of the substrate to polyubiquitin chains presumably serves to anchor the substrate to the base while it is unfolded and translocated through a gated channel into the 20 S CP for proteolysis. The lid attaches to the base and is required for proteolysis of polyubiquitin-protein conjugates (12). During this process, ubiquitin (Ub) is hydrolyzed from the substrate by proteasome-associated DUBs, enabling recycling of ubiquitin even as the substrate is degraded (11).

Almost all known DUBs are cysteine proteases that specifically hydrolyze an amide bond following the carboxyl terminus of the ubiquitin moiety (glycine 76), usually a peptide bond between this carboxyl group and an amino group on the substrate: either a lysine residue or the N terminus itself (7, 13). Other types of amide bonds such as those linking ubiquitin to small organic molecules (e.g. Ub-AMC) can also be cleaved, a property often used to design substrates for the study of deubiquitinases in vitro (14). Based on their size, sequence homology, and active sites, DUBs have classically been categorized as ubiquitin-specific proteases (UBPs) or ubiquitin C-terminal hydrolases (UCHs). For example, in Saccharomyces cerevisiae, there are at least 17 different DUBs, 16 of which are UBP-S and one that is a UCH (15). Other organisms have a greater number of DUBs from both of these classes. The MPN+ or JAMM family comprises a novel and unrelated third class of DUBs. These enzymes contain a consensus motif, EX5-HXX5-SX5-D that bears some resemblance to the active site of zinc metalloproteases and may function as tightly regulated metallopeptases (16–19). While this manuscript was under final preparation for publication, the three-dimensional structure of an archaeal MPN+ protein related to Rpn11 was reported (20).

This extremely important advancement strongly supports the MPN+/JAMM motif as a metal-binding site with a zinc-coordinated water ligand serving as the active site nucleophile in this novel class of enzymes. In addition to hydrolyzing the amide bond C-terminal to ubiquitin, certain members of the above families can cleave Ub-like domains. Thus, the UCH Yuh1 (21) or the MPN+ protein Ca5 may process the Ub-like domains of Rub1/Nedd8 (22–25), although there is evidence that these two enzymes may hydrolyze ubiquitin as well (26, 27).

Complicating matters, additional unrelated deubiquitination
enzymes are continuously being discovered. Thus, members of the ovarian tumor superfamily of proteins (OTU) may constitute a new DUB family carrying a cysteine active site (28-30). Since they bind to ubiquitin-aldehyde, this family is also called outubains (for OTU domain ubiquitin-aldehyde-binding proteins). Josephines group together spinocerebellar ataxias such as ataxin-3 and Josephine-type proteins (31). Based on their sequence, members of this family are predicted to be active against ubiquitin chains or related substrates. In yet another example, cylindromatosis proteins may represent a novel class of deubiquitinating enzymes that are highly divergent from UBP’s (32). Finally, a novel family of Ub-like proteases was identified as specific for removal of various Ub-like domains from their targets (33, 34). Although they are all cysteine proteases, UBP’s, UCHs, outubains, Josephines, and Ub-like proteases do not share overall sequence homology with one another. The ongoing identification of unrelated families of proteases for removal of ubiquitin or ubiquitin-like domains and their diverse mechanisms of action raises the possibility that additional novel deubiquitinating enzymes have yet to be discovered, some possibly even relating to proteasome function.

A number of reports indicate that the proteasome, and more specifically the 19 S RP, possesses intrinsic deubiquitinating activity (17, 18, 35-41). Furthermore, proteasomes exhibit a broad spectrum of intrinsic and associated deubiquitinating activities. For example, in some studies, this activity is ATP-dependent (17, 18, 35), whereas in others it is not (37, 39). In some cases, the activity is sensitive to cysteine modifiers such as N-ethylmaleimide (NEM), ubiquitin aldehyde, ubiquitin vinyl sulfone, or ubiquitin diol (37, 39, 40), whereas in others it is not (17, 18, 35). It is not surprising, therefore, that these activities have been matched with different proteasome components. For instance, Rpn11, the most conserved subunit among the lid subunits, is an MPN+ protein responsible for deubiquitination of certain substrates (17, 18). Nonetheless, single site mutants in the MPN+/JAMM catalytic site are viable (16), and no deubiquitination by isolated lid or recombinant Rpn11 has been reported. Thus, if deubiquitination is a fundamental property of proteasomes, it would be expected to be performed by complementary subunits. Separately, Ubp6 and its human homolog USP14 have been shown to bind to proteasomes and may be responsible for removal of the proximal ubiquitin at the base of the anchoring polyubiquitin chain (40, 41). In addition, the UCH p37a (Dm) or its homologs UCH37 (Hs) and Uch2 (Sp) can attach to the 19 S RP and may be responsible for polyubiquitin chain editing (37, 39, 42). A clear ortholog of p37a has not been identified in S. cerevisiae, suggesting that these functions are complemented by another component. An unrelated DUB, Ubp4/Doa4, has been proposed to trim short residual polyubiquitin chains from proteasome-bound substrates in this organism (43).

The exact role that deubiquitination plays in proteasome function is far from clear. Why, for example, are proteasome-associated DUBs necessary when numerous “free” ones abound? By removing the polyubiquitin anchor, can proteasome-associated DUBs provide one last proofreading attempt, a chance to salvage substrates before they are irreversibly peptide? Or must the polyubiquitin chain be removed in order to facilitate translocation of the substrate via the narrow gated channel into the proteolytic chamber of the 20 S CP? Alternatively, the emphasis may be on recycling ubiquitin and preventing it from following in the substrate’s wake. In this study, we characterized the intrinsic deubiquitinating activity of highly purified 26 S proteasomes from yeast and attempted to elucidate the mechanism by which deubiquitination intertwines with the proteasome’s main function, proteolysis. We mapped deubiquitination to both the lid and base subcomplexes of the regulatory particle. By comparing purified proteasomes from rpn11ΔH11001 and Δubp6 strains, our results point to similar contributions of Rpn11 and Ubp6, each within their respective subcomplex, with possibly yet a third unidentified proteasome-bound DUB. Surprisingly, inhibition of proteasome deubiquitinating activity promoted degradation of the monoubiquitinated test substrate along with the Ub moiety by 26 S proteasome holoenzymes but not by base-CP preparations.

**EXPERIMENTAL PROCEDURES**

**Strains**

SUB62–SUB62 (MATa his3-s3200 lys2-s801 leu2-s3, 112 trp1-1 uro3-52) was used as wild type for proteasome purifications used in Figs. 1, 6, and 7. Lid and base subcomplexes were purified from rpn11Δ strain derived from SUB62 and described previously (12, 44). Δubp6 (MY18) and its isogenic WT strain BY471 were obtained from EUROCRIB and were used in proteasome purification described in the legends to Figs. 3–7. Single site mutants in RPN11 were carried out in a strain derived from BY471 and were described previously (16). Purified proteasomes used in the remaining figures were purified from WT BY471, Δubp6, and rpn11ΔH112A.

**Δubp6 Δubp1 Double Mutant—**The RPN11 and UBPE6 loci are situated very close on the same chromosome; thus, obtaining a double mutation in both genes by mating single mutants is inefficient. To generate double mutants, we first generated a Δubp6 deletant in which HIS3 replaces the ubp6 locus (MY211) with a MX6-HIS3-MX6 cassette. Using PCR, we obtained a DNA fragment that can be used to replace WT UBPE6 with the HIS3 marker in any strain. The resulting cassette was transformed into a haploid Δrpn11 strain sustained by WT RPN11 expressed from a single copy URA3-marked plasmid. Proper genomic location of HIS3 (instead of ubp6) was verified by PCR. Various rpn11 mutants expressed from LEU2-marked plasmids were transformed into the new haploid strain. The synthetic effect of the various rpn11 mutants on Δubp6 was evaluated by plasmid shuffling using 5-fluoroacetic acid.

**Materials and Antibodies**

Ubiquitin aldehyde (Ubal) was purchased from BostonBiochem; the succinyl-LLVY-7-amido-4-methylcoumarin fluorescent peptide was from Bachem; and unless otherwise specified, all other chemicals were purchased from Sigma. Anti-green fluorescence protein (anti-GFP) antibody (Clontech) was used at a 1:5000 dilution followed by anti-rabbit horseradish peroxidase diluted 1:50,000. Anti-ubiquitin antibody (a generous gift from David Legget and Dan Finley) was used at a 1:2000 dilution, followed by anti-rabbit horseradish peroxidase diluted at 1:20000.

**Construction of Plasmids for Purification of Recombinant Proteins**

The linear Ub-GFP substrate was obtained in a multistep cloning procedure: pET19 carrying a His6-Ub-LacI-GFP (MG1) was double-digested by NcoI and XhoI and cloned into pET19 (MG1) was double-digested by NcoI and XhoI and cloned into pET19. The GFP insert was subcloned from MG32, which carried the entire open reading frame of GFP, using a double digestion of XhoI and BlpI. To obtain a His6-Ub insert without the LacI extension, we used a pGEM plasmid (MG342) double-digested with NcoI and XhoI. A one-piece ligation was performed as follows. The ubiquitin insert (from pGEM) carrying NcoI and XhoI ends and a GFP insert carrying XhoI and BlpI ends were ligated into an MG1 plasmid (pET19) doubly digested by NcoI and BlpI. Single-site substitutions replacing the methionine residue following Gly of ubiquitin with proline or replacing Gly of ubiquitin with valine were performed by PCR using a pair of mismatch primers for each mutation. The PCR products were treated by DpnI and transformed into Escherichia coli. Clones were verified by DNA sequencing and then subcloned into the E. coli strain BL21 for bacterial expression. The transformants were grown in liquid LB media supplemented with 1 mg ampicillin to A600 = 0.6–0.8 at 37°C, after which 0.1 mg isopropyl-1-thio-β-D-galactosidase was added for induction and temperature-shifted to 16°C overnight, at which time cells were harvested.

**Recombinant Proteins**

After expression in E. coli, cell lysates were clarified at 10,000 rpm for 20 min at 4°C, and the supernatant was loaded onto a Ni²⁺-
products were generated by incubation of 900 nM Ub-GFP with 8 nM purified WT proteasomes for the indicated time. Aliquots were separated by SDS-PAGE followed by immunoblotting with anti-Ub reveals that free ubiquitin is released concomitant with generation of free GFP.

In the 26 S proteasome targets the amide bond following glycine 76 of the ubiquitin moiety. Substitutions of amino acids at positions flanking the peptide bond fusing the C terminus of Ub with the N terminus of GFP are referred to here as Ub-GFP. When Ubal (45, 46) and UbG76V-GFP (45, 46) were used, which is roughly 1000-fold excess over proteasome concentration. This concentration was chosen, since lower levels of 1–5 µM Ubal used in many previous studies (17, 47) showed significant yet incomplete inhibition of deubiquitination in our studies (see Supplemental Material). We calibrated to maximal inhibition (see Supplemental Material). These levels are higher than those used in previous studies (18, 35).

Protein degradation was also followed by GFP fluorescence. Excitation of the GFP used was at 490 nm, and emission was at 510 nm. When monitoring reaction progression by GFP fluorescence, 400 µl of sample containing 5–9 nM 26 S proteasome and 700 nM Ub-GFP or GFP were incubated directly in the cuvette, and GFP fluorescence was measured.

Characterization of Intrinsic Deubiquitinating Activity by Purified Yeast 26 S Proteasomes—Previous studies have shown that polyubiquitin-protein conjugates can be deubiquitinated by the 26 S proteasome (17, 18). In order to distinguish chain trimming at the distal end from deubiquitination at the proximal ubiquitin, we designed a construct carrying a single Ub moiety fused to a target substrate. Fusion of ubiquitin to the amino terminus of proteins occurs naturally in some cases, as has been shown for MyoD, LMP1, and a few other proteins (7, 48). This concept has been incorporated successfully in other substrates previously studied for binding to or deubiquitination by the proteasome (17, 17, 18). Further, in order to distinguish between deubiquitination and degradation, we chose a highly stable protein, GFP, which cannot be easily degraded in the absence of proper targeting signals (49–52). Accumulation of processed GFP in cells expressing Ub-GFP confirms that such chimeras are efficiently recognized by ubiquitin hydrolases in vivo and are therefore bona fide substrates of the ubiquitin-proteasome pathway (49). We incubated this Ub-GFP substrate with purified WT 26 S proteasomes (45, 46) and followed the substrate’s fate. Deubiquitination was monitored by the loss of Ub and release of GFP upon the addition of purified 26 S proteasomes (Fig. 1A). Additive levels of Ub-GFP and GFP appear constant throughout the experiment, confirming that the Ub-GFP substrate is not degraded but rather efficiently deubiquitinated by purified proteasomes. Rudimen-
Proteasome Deubiquitination

DUBs are proteases that hydrolyze the amide bond following the final glycine residue at position 76 of ubiquitin. To confirm that 26 S proteasome-dependent processing of the Ub-GFP construct adheres to properties attributed to DUBs, we constructed a series of amino acid substitutions flanking the target peptide bond. Mutating glycine 76 of ubiquitin to valine (UbG76V-GFP) damages the deubiquitinating recognition sequence. Likewise, substituting the subsequent methionine (i.e. the first residue following the C terminus of Ub) to proline (Ub-Pro-GFP) also hampers the accessibility of the target amide bond, since proline confers a structural kink to the fusion substrate. Both substitutions have been used successfully to inhibit deubiquitination of various substrates in vivo (54, 55) as well as substrates used in in vitro studies in which the proximal ubiquitin was linked in a similar manner to that used in this work (17, 47). When referring generally to the various Ub-GFP fusions that we studied, we use the term Ub-X-GFP, where X represents the Met, Val, or Pro residues explained above. Indeed, both substitutions slow down the deubiquitination rate of Ub-X-GFP type substrates by purified proteasomes (Fig. 1E). This observation indicates that proteasome-dependent processing occurs specifically at the amide bond following residue 76 of ubiquitin, with a preference for the natural glycine residue at this location. Interestingly, the finding that processing of UbG76V-GFP and Ub-P-GFP does occur, albeit at a slower rate, indicates that purified 26 S proteasomes harbor a potent deubiquitinating enzyme. This should be taken into account when using similarly linked conjugates for probing the ubiquitin-proteasome pathway in vivo.

Both Rpn11 and Ubp6 Are Found in Purified Proteasomes and Contribute to Proteasome Deubiquitination—What is the identity of the subunit responsible for this DUB activity? Ubp6 had been shown to interact with Rpn1 in the base subcomplex of proteasomes, which were not subjected to high salt concentrations during affinity purification, and contribute to ubiquitin-specific hydrolytic activity (40, 41, 56). Independently, Rpn11, an integral and stoichiometric component of the lid subcomplex (12, 57), was also found to be responsible for part of the intrinsic deubiquitinating activity of proteasomes purified from various sources (17, 18). To estimate the relative contributions of Rpn11 and Ubp6 to overall deubiquitinating activity, we purified 26 S proteasomes from WT, rpn11D122A, and Δubp6 strains. Aspartate 122 of Rpn11 is part of the MPN + consensus motif; this residue has been shown to be important for proteolysis by the proteasome but not for proteasome stability. It is important to note that the single site substitution mutant rpn11D122A complements the null and is viable, albeit slow growing (16). A similar substitution at this location, rpn11D122E, was found to be nonviable (17); the differences could be due to slight effects these substitutions have on Rpn11 mechanism. Viability of rpn11D122A could reflect redundancy of Rpn11 function, or residual activity associated with this substitution. Regardless, simultaneous substitution of both histidines (the rpn11JAXA mutant) was nonviable (18). Likewise, double substitution of both these histidine residues could not rescue RNA interference treatment of DmS13/rpn11 in insect cells (58).

Proteasome preparations were resolved by SDS-PAGE, and protein bands in the region of interest were sequenced by MS-MS mass spectrometry, and their identity was confirmed. Subunit composition of standard proteasome subunits (59), their relative levels, and migration pattern were similar for proteasomes purified from all three strains, with the main difference being omission of the Ubp6 band from Δubp6 proteasomes (Fig. 2, A and B). For reasons that are unclear to us at this stage, significant levels of mitochondrial Hsp60 (CPN60/MIF4 gene product) copurified with Δubp6 proteasomes. Potentially, this could reflect pleiotropic effects of Ubp6 on mitochondrial integrity, in analogy to reports of a connection between mitochondrial function and Rpn11 (60, 61). When resolving WT proteasomes, Rpn3 and Ubp6 comigrated in 10% polyacrylamide gels, whereas only Rpn3 was visualized in proteasome samples purified from the Δubp6 strain. Nondenaturing gel electrophoresis verified that proteasomes from all three strains were intact and active (Fig. 2C). Although Ubp6 is found in purified proteasomes, its absence did not lead to detectable changes in the structure or peptide bond cleavage properties of purified proteasomes. Likewise, no gross structural change occurred in proteasomes we tested from the rpn11 mutants.

Next, we compared deubiquitinating activity of purified proteasomes to discern distinct functions for these individual subunits. 26 S proteasomes purified from either rpn11D122A or Δubp6 seem to possess approximately half the specific deubiquitinating activity of WT proteasomes when using Ub-GFP as a substrate (Fig. 3A). This is the first example of proteasome-bound Ubp6 being directly involved in deubiquitination of a ubiquitin-protein conjugate (previous studies used C-terminally modified ubiquitin such as Ub-vinyl sulfone or Ub-7-aminodimethylcoumarin). We conclude that both Rpn11 and Ubp6 contribute to the overall deubiquitinating activity of purified proteasomes, which may explain why mutations in the active site of either subunit are not lethal.

Rpn11 Is the Source of Phenantholine Sensitivity—Until recently, all known DUBs were identified as thiol proteases. Somewhat unexpectedly, deubiquitinating activity of proteasomes was found to be sensitive to metal chelators, suggesting a role for a metalloprotein in proteasome deubiquitination (17, 18, 35). Since the MPN + domain in Rpn11 is probably a metal-binding site (20), Rpn11 is thought to be the target of such inhibitors. Using previously published conditions, we calibrated the levels of the metal chelator OPA to yield maximal inhibition of deubiquitinating activity (see Supplemental Material). We found that deubiquitination of Ub-GFP by purified WT 26 S proteasomes is indeed attenuated in the presence of 10
Rpn3 and Ubp6 comigrate in these gels; thus, a fainter band representing only Rpn3 is visualized in proteasomes purified from the excised bands confirmed the identity of the following bands: Rpn1 (resolved by 10% SDS-PAGE and determined by Coomassie Blue staining. Immunoblotting combined with MS-MS mass spectrometry analysis of rpn11D122A mutant proteasomes resolved by nondenaturing PAGE (native gel) and visualized by fluorogenic peptide overlay. Samples contain both asymmetric singly capped (RP 1CP) and symmetric doubly capped (RP 2CP) proteasomes (see Ref. 45). No significant differences in gross conformation, migration pattern, or peptidase activity are observed for proteasomes purified from the three strains.

We did not observe complete inhibition of deubiquitination by lid or 26 S holoenzymes using concentrations of up to 15 mM OPA, although not abolished entirely (Fig. 3B). Proteasome conformation and general peptidase activity were minimally affected at these levels of OPA (see Supplemental Material). Higher concentrations of OPA elicited nonspecific effects on proteasome configuration and function therefore could not be used to isolate the contribution of the metalloprotease DUB from other properties of the proteasome. Nevertheless, similar results were also obtained with another zinc chelator TPEN, supporting our observations (see Supplemental Material). Insensitivity to OPA of residual deubiquitination measured for 26 S proteasomes containing Rpn11D122A (Fig. 3B, middle), points to Rpn11 as the source of the OPA sensitivity. Quite possibly, residual DUB activity observed for OPA-treated proteasomes is due to another subunit. Deubiquitination by Δubp6 26 S proteasomes, in contrast, is sensitive to OPA treatment, confirming additive contributions of Ubp6 and Rpn11 (Fig. 3B).

We did not observe complete inhibition of deubiquitination by lid or 26 S holoenzymes using concentrations of up to 15 mM OPA (see Supplemental Material), suggesting that OPA may not be an optimal metal chelator for studying Rpn11, although it was used successfully at lower concentrations in previous reports (18, 35). We conclude at this stage that at low concentrations, these widely used metal chelators do not fully inhibit deubiquitination by proteasomes, whereas at high concentrations, they may cause nonspecific effects. Nevertheless, Rpn11 can account for the bulk of heavy metal chelator-sensitive DUB activity that we do measure. The residual DUB activity that we measure in OPA-treated rpn11-mutated proteasomes indicates that in all likelihood, Rpn11 is not the sole proteasome-associated DUB.

In order to evaluate the additive roles of Rpn11 and Ubp6, we attempted to generate double mutants. A haploid Δubp6Δrpn11 strain sustained by a URA3-marked plasmid expressing WT RPN11 was transformed with LEU2-marked plasmids containing various point mutations in the MPN+ motif of rpn11. Strains deleted for both ubp6 and rpn11 that were co-transformed with plasmids encoding one of these mutants and a WT RPN11 rescue copy were unable to survive without the WT RPN11 plasmid,
indicating that a functional Rpn11 is essential in absence of Ubp6 (Fig. 4). For comparison, a mutation that leads to substitution of a highly conserved residue that is not part of the catalytic MPN+ motif, cysteine 116 to alanine, is shown in Fig. 4 to be viable in conjunction with the ubp6 null. As a control, an identical experiment carried out in Δrpn11 with plasmids expressing H111A, S119A, or D122A point mutations in Rpn11 produced viable strains (16). The synthetic phenotype associated with mutations in the ubp6 and rpn11 genes points to overlapping functions of the two-proteasome subunits they encode.

**Linking Deubiquitination to Degradation—Inhibiting proteolytic activity enhances deubiquitination of substrates by the proteasome (18). But what about the reverse process? Does inhibition of the proteasome-based deubiquitinase impose a similar stimulatory effect on proteolysis? Ubal is a specific inhibitor of known deubiquitinating enzymes (62). Recognized via its ubiquitin domain, the aldehyde group of Ubal then modifies the active site cysteine of the enzyme, thereby preventing the enzymes from binding additional ubiquitin moieties (26, 62–65).

Treatment of proteasomes with 5 μM Ubal significantly inhibits deubiquitinating activity, pointing to an important role for a cysteine-based deubiquitinase in processing Ub-GFP (Fig. 5A). Astonishingly, inhibition of deubiquitination in this manner leads to degradation of the Ub-GFP substrate. As a positive control, untreated proteasomes deubiquitinate the substrate with no significant degradation detected under similar conditions (Fig. 1). These results indicate that inhibition of deubiquitination can stimulate degradation of a monoubiquitinated substrate. In another unexpected result, Δubp6 26 S proteasomes are similarly sensitive to Ubal treatment (Fig. 5A, right). This further implies that an additional subunit sensitive to cysteine modifiers may contribute to intrinsic deubiquitination by the 26 S proteasome. To confirm our observations, we used an additional cysteine modifier, NEM, a general modifier of active site cysteines and an inhibitor of most known DUBs (62, 63, 66). We preincubated purified 26 S proteasomes with 10 mM NEM and then added the Ub-GFP substrate (for calibration, see Supplemental Material). In accordance with Ubal treatment, deubiquitinating activity of proteasomes was also inhibited by NEM, once again promoting degradation of the test substrate (Fig. 5B). Immunoblotting with anti-Ub confirms that the substrate is not processed into Ub and GFP domains (as in untreated proteasomes; see Fig. 1) but rather proteolyzed entirely together with the ubiquitin moiety (Fig. 5C).

To confirm that Ub-GFP is indeed proteolyzed by DUB-inactivated proteasomes, we followed the fate of the substrate by fluorescence. Fluorescence intensity of GFP can be used as an indicator of its tertiary structure, since processed or unfolded GFP loses its intrinsic fluorescent properties (52). No decrease in GFP fluorescence was measured when Ub-GFP was incubated with proteasomes, indicating that overall levels of GFP in the reaction solution remain constant (Fig. 6A, left). Samples taken during the time course of this experiment verify that the Ub-GFP is in fact deubiquitinated, although additive levels of Ub-GFP and released GFP remain constant (Fig. 1). In contrast, a consistent decrease in substrate fluorescence is measured upon incubation of Ub-GFP with NEM-treated proteasomes (Fig. 6A), substantiating our observation that the substrate is degraded under these conditions (Fig. 5). These observations demonstrate that inhibition of deubiquitination can promote degradation of a monoubiquitinated substrate along with the ubiquitin tag.

Degradation of Ub-GFP constructs by NEM- or Ubal-pretreated proteasomes, but not by untreated proteasomes, raises the remote possibility that proteolysis is the outcome of a change in proteasome configuration that makes it less regulated and more competent for proteolysis. To elucidate this point, we wished to test whether degradation is dependent on the ubiquitin tag. Untagged GFP is not proteolyzed by treated or by untreated proteasomes (Fig. 6, B and C). Thus, targeting of the stable GFP substrate is directly due to the ubiquitin tag, and degradation would appear to be a direct outcome of the inability of cysteine-modified treated proteasomes to adequately remove the ubiquitin tag, prior to unfolding and translational movement of the substrate into the 20 S CP for proteolysis.

**Participation of the Lid and Base Subcomplexes in Proteolysis and Deubiquitination**—Since we found that both Rpn11 and Ubp6 contribute to proteasome degubiquitination, we wished to map their activity to subcomplexes of the 26 S proteasome. We compared inherent deubiquitinating activities of purified 26 S holoenzymes, lids, base-CPs (“lidless proteasomes”; see Ref. 12), and 20 S CPs (Fig. 7A). Both “lidless” proteasomes and lid subcomplexes were able to deubiquitinate Ub-GFP, yet they exhibited lower specific activities compared with 26 S holoenzymes. Importantly, no peptidase activity was measured for purified lid, substantiating our observation that deubiquitination is a distinct process from proteasome peptidase or protease properties. To the best of our knowledge, this is the first direct demonstration that lids are potent for deubiquitination, since previous studies did not detect activity with isolated lid subcomplexes (18, 41). Another important observation is that we did not detect deubiquitination or degradation of Ub-GFP upon incubation with the 20 S CP alone (Fig. 7A), although these 20 S CP samples were competent for peptidase activity. The deubiquitinating ability of base-CP is thus attributed to the base subcomplex. To conclude, the intrinsic deubiquitinating activity of the 26 S proteasome is localized to two distinct subcomplexes of the 19 S RP, suggesting that they emanate from discrete subunits.

As mentioned above, the natural candidates for deubiquitinating activity would be Rpn11 in the lid and Ubp6 in the base.

![Fig. 4. Synthetic phenotype for ubp6 and rpn11 double mutants.](Image 74x607 to 290x738)
Fig. 5. Inhibition of proteasome deubiquitination by cysteine modifiers promotes degradation. A, effect of Ubal. 8 nM conventionally purified 26 S proteasome preparations were pretreated with 5 μM Ubal, followed by the addition of Ub-GFP to a final concentration of 500 nM; products were resolved by 12% SDS-PAGE and immunoblotted for GFP. In contrast to the reaction with untreated proteasomes (Fig. 1), Ub-GFP was degraded, and no free GFP was released. B, effect of NEM; similar reaction as in A but in the presence of 10 mM NEM. Reaction products were monitored by blotting with anti-GFP. C, fate of Ub; same as in B, but products were resolved by 18% SDS-PAGE and immunoblotted with anti-Ub. No free ubiquitin was detected, indicating that Ub-GFP was hydrolyzed in its entirety along with the Ub domain (compare with Fig. 1B in the absence of inhibitors).

As such, lid deubiquitination would be expected to be sensitive to metal chelators, whereas deubiquitination by the base should be sensitive to cysteine modifiers. As expected, no effect of OPA was detected on deubiquitination by the base (Fig. 7B, top right). Deubiquitination by free lid was inhibited to a limited extent (middle). Incubation of 26 S holoenzymes with 10 mM OPA is shown for comparison (Fig. 7B, left) and confirms results shown in Fig. 3 demonstrating that deubiquitination by the 26 S proteasome is only partially sensitive to the presence of 10 mM OPA under these conditions. Incubation with NEM, on the other hand, completely inhibits deubiquitination by both base and 26 S holoenzymes (Fig. 7B, bottom). As expected, no effect is observed on the enzymatic properties of the lid. This result is in complete agreement with deubiquitination in the base being attributed to a cysteine protease and a "cryptic" protease situated in the lid. A fascinating aspect of proteasome mechanisms was unveiled when inhibition by NEM promoted degradation of the test substrate by proteasome holoenzymes (left) but not by base-CPs (right). This result confirms a previously published result (12) showing that the lid was required for degradation of a polyubiquitinated substrate, whereas an unfolded substrate could be degraded by base-CP complexes independent of a ubiquitin tag.

Proteasomes and base-CPs that we prepared retained Ubp6, whereas lid and 20 S CPs did not contain detectable levels of Ubp6 (Fig. 7C). Although the subunit compositions of base and lid subcomplexes we used were identical to a published preparation (12), the presence of Ubp6 in the 19 S RP or base samples was not detected previously, probably due to comigration of Ubp6 and Rpn3 in SDS gels (see Fig. 2A). Our results also indicate that Ubp6 can copurify with base subcomplexes purified by conventional chromatography (i.e. not by affinity chromatography (41)), despite undergoing salt elution during the purification procedure. Based on these results, Ubp6 behaves like an integral component of the 19 S RP, tightly bound to the base. The hydrolytic properties of Ubp6 have been reported to be activated upon lid attachment to the base, implying a transient interaction between Ubp6 and the lid as well (41). This may explain why base-CP complexes appear to contain slightly lower molar ratios of Ubp6 compared with 26 S holoenzymes (Fig. 7C). Since Ubp6 does not copurify with lid, the interaction between the two is probably transient, supporting the localization of Ubp6 to the base (41).

DISCUSSION

A little over 20 years ago Hershko et al. (67) observed that ubiquitin conjugation is a reversible process and predicted the existence of specific amidases for hydrolysis of the amide bond linking glycine 76 of ubiquitin with an amino group on the substrate. These deubiquitinating enzymes (also referred to as DUBs or isopeptidases) in effect compete with the proteasome that acts on the conjugated form of the substrate. Myriad such DUBs have since been identified. In analogy to different E3 ligases that conjugate ubiquitin to subsets of substrates, certain DUBs may be specific for distinct ubiquitin-substrate configurations, hence their variability. Nonetheless, many probably carry out overlapping functions, since none of the 17 known cysteine-based DUBs in S. cerevisiae is essential (15), and mutations in the active site of Rpn11 are viable (16). In an interesting development, Hershko and co-workers (35) later identified a deubiquitinase activity associated with the 26 S proteasome. The authors proposed that this activity releases the ubiquitin anchor following irreversible threading of the substrate into the 20 S CP; otherwise, premature dissociation of the substrate could occur. A wide range of ubiquitin-hydrolase activities associated with the proteasome has since been reported. The current study provides direct documentation that deubiquitination activity is localized simultaneously to both lid and base subcomplexes of the proteasome and that each can function independently on a monoubiquitinated substrate (Fig. 7).

Rpn11 and Ubp6, each within a separate subcomplex, play complementary although not fully overlapping roles (Figs. 3–5). Ubp6 is localized to the base, even in proteasomes puri-
fluorescence upon the addition of proteasomes. 700 nM Ub-GFP was
substrate. Indeed, deletion of ubiquitin might be wastefully degraded along with the target sub-
role may be recycling of ubiquitin; unless it is removed, ubiquitination of
the fused proximal ubiquitin in these examples. An additional
deubiquitination of
Rpn11 to deubiquitination remains somewhat
difficult in the cytoplasm. The contribution of the lid subunit
strain, ubiquitin might be degraded and therefore less abun-
dant in the surface of NEM. 500 nM purified GFP was incubated with 6 nM purified 26 S
proteasomes (as in Fig. 5). Note that untagged GFP is not degraded
right addition of substrate (right). A steady decrease in GFP fluorescence is
observed, corroborating the observation that GFP is hydrolyzed by
proteasomes (as in Fig. 5). Note that untagged GFP is not degraded
under similar conditions (see Supplemental Material). B, GFP is inert
to the addition of purified 26 S proteasomes in the presence or absence
of NEM. 500 nM purified GFP was incubated with 6 nM purified 26 S
proteasomes in the presence (right) or absence (left) of 10 mM NEM. No
changes in real time fluorescence of GFP were measured. C, 500 nM
GFP was incubated with 8 nM untreated (right) or NEM-treated (left)
purified 26 S proteasomes. Reaction products were resolved by 12%
SDS-PAGE and immunoblotted for GFP. No degradation of GFP was
detected under these conditions either by untreated or by NEM-treated
proteasomes. The fate of GFP can be compared with degradation of
Ub-GFP that was measured under similar conditions by NEM-treated
26 S proteasomes (Fig. 5). These results confirm results presented in
the Supplemental Material showing that degradation of Ub-GFP is
dependent on the ubiquitin tag. Untagged GFP is not a substrate for the
proteasome.

**Fig. 6.** Monitoring degradation of Ub-GFP conjugates. A, GFP fluorescence upon the addition of proteasomes. 700 nM Ub-GFP was
incubated with 6 nM purified 26 S proteasomes, and changes in GFP
fluorescence were recorded. Despite being deubiquitinated throughout
the course of this reaction (see Fig. 1), fluorescence intensity of Ub-GFP
remained unchanged, pointing to constant levels of total GFP (left panel). The reaction was repeated under similar conditions but with a
proteasome sample that was preincubated with 10 mM NEM before the
addition of substrate (right). A steady decrease in GFP fluorescence is
observed, corroborating the observation that GFP is hydrolyzed by
proteasomes (as in Fig. 5). Note that untagged GFP is not degraded
under similar conditions (see Supplemental Material). B, GFP is inert
to the addition of purified 26 S proteasomes in the presence or absence
of NEM. 500 nM purified GFP was incubated with 6 nM purified 26 S
proteasomes in the presence (right) or absence (left) of 10 mM NEM. No
changes in real time fluorescence of GFP were measured. C, 500 nM
GFP was incubated with 8 nM untreated (right) or NEM-treated (left)
purified 26 S proteasomes. Reaction products were resolved by 12%
SDS-PAGE and immunoblotted for GFP. No degradation of GFP was
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Ub-GFP that was measured under similar conditions by NEM-treated
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the Supplemental Material showing that degradation of Ub-GFP is
dependent on the ubiquitin tag. Untagged GFP is not a substrate for the
proteasome.

A distinctive property of purified proteasomes is that they are
competent to deubiquitinate Ub-Pro or UbG76V fusions (Fig. 1). In
accord, ∆ubp6 has been shown to stabilize Ub-Pro-β-galactosidase in vivo (41), indicating that Ubp6 may participate in
deubiquitination of “difficult” substrates or in the removal of the
fused proximal ubiquitin in these examples. An additional role may be recycling of ubiquitin; unless it is removed, ubiquitination might be wastefully degraded along with the target substrate. Indeed, deletion of ubp6 was reported to bring about a
noticeable decrease in levels of free ubiquitin (15, 41). In this strain, ubiquitin might be degraded and therefore less abundant in the cytoplasm. The contribution of the lid subunit Rpn11 to deubiquitination remains somewhat “cryptic.” In light of its distal positioning within the lid and away from the
surface of the α ring through which unfolded substrates are
translocated (68, 69), Rpn11 is anticipated to partake in trimming or editing of polyubiquitinated chains (17, 18). Neverthe-
less, mutants in the active site of Rpn11 are conditional lethal with a deletion of ubp6, suggesting that there is significant overlap in their functions (Fig. 4). That ∆rpn11 is lethal, whereas UBp6 is a nonessential gene, may be due to additional roles of Rpn11 in proteasome stability. For example, a mutation at the noncatalytic C terminus of Rpn11 promotes disso-
ciation of the lid (18). Rpn11 also plays pleiotropic roles in
maintaining the function and intact structure of mitochondria that as far as we know cannot be explained solely by the deubiquitinating properties of Rpn11 (60, 61). These points should be addressed in future studies.

The crystal structure of an archaeal MPN+/βJAMM protein was published while this work was under review, identifying a zinc ion liganded to three key MPN+ motif residues (His, His, and Asp) with a water or hydroxide molecule as the fourth ligand, making it extremely plausible that members of this family are indeed hydrolytic enzymes (20). Thus, finding both
Rpn11 and Ubp6 in purified proteasomes accounts for its sen-

sitivity to both cysteine and metalloprotein modifiers (Figs. 3, 5, 6, and 7). The lid itself exhibits properties similar to the "cryptic" metalloprotease activity that was attributed to Rpn11 (17, 18). Indeed, a single site mutation in the proposed metal binding site of Rpn11 (the above mentioned Asp residue) desensitizes proteasomes to the metal chelator OPA (Fig. 3). In contrast, cysteine modifiers such as NEM and Ubal completely inhibit deubiquitination of a monoubiquitinated substrate by base-CP or by 26 S holoenzymes (Fig. 7), in agreement with a cysteine-based DUB situated in the base subcomplex. Unexpectedly, deubiquitination by purified proteasomes lacking Ub\textsubscript{p} is still sensitive to Ubal and NEM (Figs. 5 and 6), raising the possibility that yet another thiol deubiquinase associates with proteasomes. Finding multiple DUBs from divergent families intimately associated with proteasome preparations portrays the proteasome as a self-contained entity able to perform multiple deubiquitination tasks.

Inhibition of proteasome deubiquitination stimulates proteolysis of the monoubiquitinated substrate that we tested (Figs. 5–7). In a similar example, Ub-OM (Ub fused to the unfolded ovomucoid moiety) was shown to be a substrate for both deubiquitination and degradation by the proteasome (17). It should be pointed out, however, that untagged ovomucoid alone was proteolyzed almost as efficiently. GFP used in the current study presumably resembles natural substrates, which are usually stable, tightly folded proteins that are not proteasome substrates unless ubiquitinated. In contrast to GFP, Ub-GFP is recognized by the proteasome and rapidly deubiquitinated and released (Figs. 1 and 7). Without the ubiquitin anchor, proteasome-substrate interaction is transient, and GFP is rescued. Thus, rapid deubiquitination circumvents proteolysis. In this scenario, deubiquitination of short chains (apparently in contrast to longer chains) occurs before irreversible translocation of the substrate into the proteolytic channel. This result supports a previous proposal that longer chains (polyubiquitin) serve as "timers"; by slowing down chain disassembly, unfolding and proteolysis are promoted over substrate release (36, 47). In the case of Ub-OM, \( k_{\text{cat}} \) decreased from 1.2 to 0.12 min\(^{-1} \) when Ub-OM was changed to Ub\textsuperscript{G76V}-OM, the difference reflecting the need to degrade Ub in addition to the unfolded OM moiety. Interestingly, in the case of Ub-GFP, deubiquitination and degradation rates of proteasomes purified from yeast are almost identical (Figs. 1 and 5). In fact, a rough estimate of the degradation rate in Fig. 5 yields a turnover number of 2 min\(^{-1} \), a remarkably fast rate, considering that the proteasome degrades not only the well folded GFP but also the highly stable ubiquitin molecule. We hypothesize that under normal conditions, disassembly of a polyubiquitin chain would be slower, providing sufficient time for unfolding and degradation to occur.

The turnover rates of deubiquitination by purified proteasomes and degradation rates by NEM-treated proteasomes are remarkably similar (Figs. 1 and 5). As far as we understand, these results point to an upstream rate-limiting step that is common to both deubiquitination and proteolysis processes. This upstream step is most likely related to recognition and binding of the Ub-conjugate in a proper configuration. Support for such an idea can be taken from the structural basis of a ubiquitin C-terminal hydrolase mechanism. The crystal structure of Yuh1 shows major reorganization upon ubiquitin binding, with a fascinating polypeptide loop clamping down on the ubiquitin substrate after it enters the active site (26). The importance attributed to anchoring substrates may account for some of the differences between the results presented above and previous studies with differently conjugated substrates. For example, recognition and binding sequences of a polyubiquitinated substrate (18) or a monoubiquitinated yet unfolded substrate (17) could be significantly different from Ub-GFP.

In many cases, anchoring polyubiquitin chains to the proteasome has been mapped directly to the base or to auxiliary factors that in turn interact with the base (44, 70–74). Even so, the lid is also required for proteolysis of both polyubiquitinated (12) and monoubiquitinated (Fig. 7) substrates. The role of the lid in deubiquitination alone does not satisfactorily account for this constraint, since inhibition of the lid component Rpn11, whether chemically or mutationally, only partially affects proteasome function (Fig. 3). It should be emphasized that a few loosely folded substrates that are not ubiquitinated can be degraded in the absence of lid (12) or by naturally lidless archaean proteasomes (75, 76). Perhaps the lid plays additional roles in interacting with polyubiquitin chains.

Modification by monoubiquitin is generally considered to target proteins to the lysosome or vacuole for degradation (77). In contrast, polyubiquitination stimulates degradation of target proteins by the proteasome but may not be an absolute requirement (78). In accord, monoubiquitination (including ubiquitin-protein fusions) has been reported to be sufficient for breakdown of certain substrates by the proteasome (17, 35, 37, 47, 64, 79, 80). We find that GFP can be targeted by a single ubiquitin moiety to the proteasome and degraded. This result indicates that not all substrates need a polyubiquitin chain to be efficiently targeted to the proteasome. Possibly, ubiquitin may not even be an absolute requirement, since GFP can be recognized by the proteasome, unraveled, and degraded by various nonubiquitin tags, such as a 37-amino acid sequence from the C terminus of ODC (50), an unfolded polypeptide domain (51), or, in the case of archaean PAN-20 S CP complexes, by the short ssrA sequence (75, 76). The uniqueness of ubiquitin may actually lie in its being a reversible tag. The plethora of cellular DUBs may serve as proofreading devices allowing for reversal of fortune at various stages of the process from ubiquitination to degradation. Precisely for this reason, some polyubiquitin signals may have to be protected in order to serve as adequate targeting signals until they reach the proteasome (81, 82). It has been predicted that polyubiquitin chains commit a substrate to unfolding and degradation by the proteasome, whereas short chains are poor substrates because they are "edited" by DUBs (36, 47, 84). Upon inhibition of intrinsic proteasome deubiquitination, a monoubiquitinated substrate can transform into an efficient proteasome substrate.

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