Specificity of the Ubiquitin Isopeptidase in the PA700 Regulatory Complex of 26 S Proteasomes

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The specificity of the ubiquitin (Ub) isopeptidase in the PA700 regulatory complex of the bovine 26 S proteasome was investigated. Disassembly of poly-Ub by this enzyme is restricted to the distal-end Ub of the substrate, i.e. the Ub farthest from the site of protein attachment in poly-Ub-protein conjugates. The determinants recognized by the isopeptidase were probed by the use of mutant ubiquitins incorporated into Lys48-linked poly-Ub substrates. PA700 could not disassemble poly-Ub chains that contained a distal Ub(L8A,I44A). This suggested either that the enzyme interacts directly with Leu8 or Ile44 or that it recognizes a higher order structure that caps the distal end of a poly-Ub substrate and is destabilized by Ub(L8A,I44A). The previously determined di-Ub crystal structure (Cook, W. J., Jeffrey, L. C., Carson, M., Chen, Z., and Pickart, C. M. (1992) J. Biol. Chem. 267, 16467–16471) offered a candidate for such a “cap.” In solution, however, this structure was not observed by 1H NMR spectroscopy. This and the finding that di-Ub with a single proximal Ub(L8A,I44A) is readily cleaved suggests that Leu8 and Ile44 in the distal-end Ub contact the isopeptidase directly. In addition to Lys48-linked chains, PA700 also could disassemble Lys6- and Lys11-linked poly-Ub, but, surprisingly, not α-linked di-Ub. Results with these and other substrates suggest that specificity determinants for the PA700 isopeptidase include Leu8, Ile44, and Lys48 on the distal Ub and, for poly-Ub, some features of the Ub–Ub linkage itself.

In ubiquitin (Ub)-mediated proteolysis, the attachment of Ub marks a protein for ATP-dependent degradation by the 26 S proteasome complex. This pathway has emerged as the major system of non-lysosomal intracellular proteolysis in eukaryotes (reviewed in Refs. 1 and 2). Ub is a highly conserved 76-residue protein that is found both free and as a covalent adduct with other proteins. Ub-protein conjugation (“ubiquitination”) is catalyzed by multiple enzymes that ultimately form an isopeptide bond that links the C-terminal α-carboxylate of Ub to an ε-amino of a lysine within the protein substrate. Many molecules of ubiquitin can be conjugated to a single protein target (3), and a distinction can be made between substrates with multiple lysines that are ubiquitinated and those in which multiple ubiquitins are elaborated from a single lysine via secondary Ub–Ub isopeptide linkages (4). This latter “poly-Ub” structure has been implicated as an essential determinant for efficient recognition by the 26 S proteasome of many (4, 5), but perhaps not all (3, 6, 7), Ub-dependent degradation substrates. Depending upon the particular Ub-conjugating enzyme and substrate involved, a variety of Ub–Ub linkages can be found in poly-Ub chains. Typically, the poly-Ub chains on substrates for degradation are linked via the Lys48 side chains of the ubiquitins (4, 5), but there is biochemical and genetic evidence that Ub–Ub isopeptide linkages also are made through lysines 6, 11, 29, and 63 (8–11).

Whether a protein is degraded by the Ub system has been thought to depend primarily on recognition by Ub-conjugating enzymes. However, the existence of a large family of deubiquitinating enzymes (“isopeptidases”) offers additional opportunities for the regulation of substrate turnover. On the one hand, deubiquitinating enzymes may selectively down-regulate the degradation of a limited subset of ubiquitinated proteins through interactions that are influenced by substrate identity. Such a role has been suggested for the enzymes encoded by the Drosophila fat facets and murine DUB-1 genes (12, 13). In contrast, other deubiquitinating enzymes appear to promote proteolysis in a generalized fashion by preventing the accumulation of degradation products derived from poly-Ub chains (14, 15). An isopeptidase whose activity depends solely upon the structure of the substrate-linked poly-Ub chain(s) might also down-regulate degradation selectively, but in a manner that does not depend upon the properties of the substrate moiety. This general idea, which we will refer to as “editing” by an isopeptidase, has been discussed by Ellison and Hochstrasser (16) and, in an early form, by Hershko et al. (17).

Evidence of editing by isopeptidases recently has been described. When added to a reticulocyte lysate, low concentrations of the isopeptidase inhibitor Ubal were found to enhance the Ub-dependent degradation of α-globin, a poor ubiquitination substrate, but not other, more efficiently ubiquitinated proteins (18). The results from this and a related study (19) were interpreted in terms of the kinetic partitioning of poly-Ub-protein or Ub-protein conjugates between two fates: degradation by the 26 S proteasome and rescue of the protein via deubiquitination by an editing isopeptidase. A candidate for an
editing isopeptidase has been found within the PA700 (19 S) regulatory complex of bovine 26 S proteasomes (20). This enzyme, which is a tightly bound, stoichiometric component of the PA700 complex, was found to promote the selective rescue of poorly ubiquitinated proteins from degradation by reconstituted 26 S proteasomes in vitro. The PA700 isopeptidase is uniquely specific for the distal (i.e. growing) ends of Lys48-linked poly-Ub chains, and this property may help bias degradation by the 26 S proteasome to highly ubiquitinated conjugates (20).

In this paper, we have probed the PA700 isopeptidase activity with a variety of wild-type and mutant poly-Ub substrates to understand the specificity for the distal Ub of poly-Ub chains. In particular, the potential roles of Leu8 and Ile44, Ub residues that are known to be important for poly-Ub binding to the 26 S proteasome (21), were investigated. We considered whether recognition by the isopeptidase involves a structure similar to that determined crystallographically for Lys48-linked poly-Ub (22); the distal end of a poly-Ub chain could be "capped" with such a structure, which might be recognized by the isopeptidase. To address this possibility, we have used two-dimen-sional 1H NMR methods to examine the structure of di-Ub in solution, and di-Ub derivatives designed to destabilize the conformation of this hypothetical cap were tested as substrates. We also examined the ability of the PA700 isopeptidase to cleave α-linked di-Ub and poly-Ub conjugates that contain isopeptide linkages through Lys8 or Lys11.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine Ub, creatine phosphokinase (type III), yeast hexokinase (type C-300), and yeast inorganic pyrophosphatase were purchased from Sigma, and bovine histone H2b was from Boehringer Mannheim. Affinity-purified Ub-activating enzyme (E1) was prepared from bovine red blood cells or rabbit liver (23, 24); recombinant E225K was purified after its expression in Escherichia coli (25); and recombinant E225K and Rad6p-conjugating enzymes were provided by A. Haas (Wisconsin College of Medicine, Milwaukee, WI). The bovine PA700 complex was isolated as described previously (26). Ub(K48R), Ub(L8A), Ub(L8A), and Ub(L8A, I44A) were purified after expression in E. coli as described (4, 21). Ub(L8W) was made similarly. Ubdiol was synthesized by carboxypeptidase Y-catalyzed exchange of (2S)-3-amino-1,2-propanediol for Gly in Ub and was purified by cation-exchange chromatography2; the C-terminal diol derivatives of mutant forms of Ub (see below) were made in a like manner. Ub was produced from Ubdiol by periodate oxidation (27). Radiolabeling of proteins with carrier-free 125I was done as follows. The conjugation reaction (90 min) contained 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 5 mM dithiothreitol, 0.5–40 μM di-Ub, and 8–80 μM PA700. Incubations were carried out at 37 °C for various times up to 1 h, and the reactions were stopped by additions of equal volumes of 1M acetic acid. Substrates and products were separated by cation-exchange HPLC (TSK SP-NPR column eluted at 1.25 ml/min with 0.05–0.45 M NaCl in 25 mM NH4OAc, pH 4.5) and quantified by their fluorescence as described above.

**Determination of Kinetic Parameters for the Disassembly of Di-Ub Substrates by the PA700 Isopeptidase**—Saturation curves for the disassembly of nonfluorescent di-Ub derivatives to Ub (and, depending on the substrate, Ubdiol) were determined as follows. Reaction mixtures (50–300 μl) contained 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 5 mM dithiothreitol, 0.5–40 μM di-Ub, and 8–80 μM PA700. Incubations were carried out at 37 °C for various times up to 1 h, and the reactions were stopped by additions of equal volumes of 1M acetic acid. Substrates and products were separated by cation-exchange HPLC (TSK SP-NPR column eluted at 1.25 ml/min with 0.05–0.45 mM NaCl in 25 mM NH4OAc, pH 4.5) and quantified by their absorbance at 235 nm; all Ub and Ubdiol species were assumed to have identical extinction coefficients. For studies of inhibition by Ub or Ubdiol, Ub–Ub(L8A, I44A)diol was used as the substrate, and the reactions were monitored by quantifying either the released Ub(L8A, I44A)diol or the released di-Ub. This substrate was chosen because one of the two products always could be resolved by HPLC from the exogenously added Ub or Ubdiol inhibitor. Measured velocities were converted to molecules of substrate processed per enzyme-min and are based on a single isopeptidase active site in each 700-kDa PA700 complex (31).

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Restrict it to the proximal position of the chain. In this manner, a Lys48-linked trimer consisting of mutants and fluorescently labeled ubiquitins was made to have the sequence Ub(L8A, I44A)-Ub(L8A, I44A)-Ub(L8W, L8W, L8W). Because Ub(L8A, I44A) retained the C-terminal carboxylate, there was concomitant formation of homopolymeric Ubal, which had been preincubated at 37 °C for 20 min. To prevent further synthesis of poly-Ub conjugates during the disassembly assay, EDTA or hexokinase + glucose were used to inhibit the ATP-dependent activation of Ub by E1. To 40 μl of the conjugation reaction was added 20 μl containing 16 μM hexokinase and 4 mM glucose; to another original reaction was added 20 μl of 10 mM EDTA, and both mixtures were incubated at 37 °C for 10 min. For the disassembly assay, each of the two 60-μl reaction mixtures was further divided into aliquots of 15 μl. One aliquot was used as the "no PA700" control; to another was added 2 μl of 0.44 mg/ml PA700. To a third aliquot was added 2 μl containing 0.44 mg/ml PA700 and 2 μM Ub, which had been preincubated at 37 °C for 20 min.
RESULTS

Ub Residues Leu^8^ and Ile^44^ Are Involved in Substrate Recognition by the PA700 Isopeptidase—We have examined whether Leu^8^ and Ile^44^, residues on Ub that affect the targeting of conjugates to the 26 S proteasome (21), also are required for recognition by the Ub isopeptidase in the PA700 regulatory complex of the 26 S particle. Leu^8^ and Ile^44^ are part of the hydrophobic patch on the surface of Ub (38) and are essential for the interaction between poly-Ub chains and S5a, a poly-Ub-binding protein in the 26 S proteasome (21). We had shown previously that the PA700 isopeptidase disassembles Lys^48^-linked poly-Ub chains specifically from the distal ends of these substrates (20). By the incorporation of Ub(L8A,I44A) into the distal position (see Fig. 1 legend for definitions of distal and proximal Ub) of fluorescent LY-labeled poly-Ub chains, we developed substrates to test whether Leu^8^ or Ile^44^ is involved in processing by the PA700 isopeptidase. Assays of PA700 isopeptidase activity with the wild-type and mutant LY-labeled tri-Ub substrates indicated that Ub(L8A,I44A) resists cleavage (Fig. 2). These results implicated Ub residue Leu^8^ or Ile^44^ (or both) as important for substrate recognition by the PA700 isopeptidase. Assays that compared LY-labeled di-Ub substrates that contained Ub or Ub(L8A,I44A) at the distal position similarly showed that the altered Ub prevented cleavage.

The effects of the L8A and I44A mutations on PA700 isopeptidase activity are not likely to be due to an altered Ub tertiary structure. The Leu^8^ and Ile^44^ side chains are exposed at the Ub surface (38, 39), and both the singly and doubly modified ubiquitins can be activated and conjugated to proteins (this work and Ref. 21). Thus, our results with Ub(L8A,I44A) most likely reflected the specificity of the PA700 isopeptidase rather than a global unfolding of individual Ub units within poly-Ub chains. We next considered two other explanations as to why the PA700 isopeptidase cannot process these altered poly-Ub chains. Possibly, Leu^8^ or Ile^44^ interacts directly with residues on the isopeptidase. Alternatively, as is evident from the crystal structure of Lys^48^-linked di-Ub (22), these same hydrophobic residues can stabilize a unique and compact structure of the substrate in such a conformation may be required for recognition and cleavage of the distal-end Ub by the PA700 isopeptidase. This latter possibility was addressed by the studies described below.

The Conformation of Di-Ub Determined Crystallographically Is Not Observed for the Protein in Solution—In the di-Ub crystal structure, Leu^8^, Ile^44^, and Val^70^ from each Ub are found within a hydrophobic core that is bisected by an approximate 2-fold symmetry axis. The stability of this structure, depicted...
Fig. 3. As determined by x-ray crystallography, Lys48-linked di- and tetra-Ub adopt different conformations. A, the crystal structure of di-Ub (22) reveals a conformation with pseudo 2-fold symmetry. This structure is stabilized by hydrophobic interactions of side chains from Leu8, Ile44, and Val70 contributed by each Ub and buried within the Ub-Ub interface. Side chain atoms for these residues are represented by the light (Leu8), medium (Ile44), and dark (Val70) spheres; note that, in this view, most of the Val70 side chain atoms are occluded by Leu8. B, the crystal structure of tetra-Ub (40) shows a molecule with a 2-fold screw axis of symmetry. This conformation is stabilized principally by multiple electrostatic interactions between ubiquitins; one-half of the tetramer is depicted. As with the Ub monomer (38), the side chains of Leu8, Ile44, and Val70 (indicated by the light, medium, and dark spheres, respectively) are exposed at the protein surface. In A and B, the distal Ub is on the left, and an arrow indicates the isopeptide linkage to the Lys48 side chain. The structures were drawn with Molscript (41).

in Fig. 3A, is thought to be due in part to the nonpolar side chain interactions and exclusion of solvent at this interface (22), whereas Leu8, Ile44, and Val70 are exposed to solvent in the Ub monomer (38, 39). Although the di-Ub crystal structure offered the first indication of a role for these residues, whether this conformation would be propagated through longer poly-Ub chains was unclear. Subsequently, a crystallographic study of Lys48-linked tetra-Ub showed that the di-Ub conformation was not conserved and that the contacts between ubiquitins differed drastically in the dimer and tetramer (40). In particular, the side chains of Leu8, Ile44, and Val70, which are buried in the di-Ub crystal, are exposed to solvent in the tetra-Ub crystal (Fig. 3B). This difference is a consequence of rotations within the isopeptide linkages that join the ubiquitins. As a result, the orientation of each Ub within the tetramer is stabilized by contacts with two adjacent ubiquitins. For the two internal ubiquitins in tetra-Ub, these contacts all originate within the tetramer. In contrast, for the distal- and proximal-end ubiquitins, half of the contacts are contributed by neighboring molecules in the crystal lattice. In dilute solution, these intermolecular interactions would be lost. Given the flexibility of the Ub–Ub isopeptide linkage, in solution, the two ubiquitins at the distal end of a poly-Ub chain could adopt the conformation exhibited by the di-Ub crystal structure. For the PA700 isopeptidase, specificity for the distal end of poly-Ub might involve the recognition of structural elements unique to this “di-Ub conformation.” Presumably, this conformation would be destabilized by the L8A and I44A mutations.

We have employed two-dimensional 1H NMR spectroscopy to look for evidence of the di-Ub crystal conformation for di-Ub in solution. Comparison of the crystal structures of mono-, di-, and tetra-Ub shows that the distinctive features of the di-Ub structure include the hydrophobic interface between ubiquitins and new hydrogen bonds that involve amide hydrogens of Gln49 and Leu71 on the proximal Ub. In DQF-COSY experiments to compare Ub and di-Ub, we were unable to detect any significant chemical shift changes for these amide hydrogen resonances. Similarly, the chemical shifts for side chain hydrogen atoms of Leu8, Ile44, and Val70 in di-Ub were nearly identical to those observed previously for the Ub monomer by others (42, 43) and confirmed here (Fig. 4, compare A and C). We note, however, that the 1H resonances from some of the Leu8 and Ile44 side chain atoms in di-Ub show small but reproducible chemical shift changes relative to those from the monomer; this observation is addressed further under “Discussion.” Although the few chemical shift changes listed in Table I presumably are reporting structural differences between Ub and di-Ub (see “Discussion”), the bulk of the chemical shift data suggests that the average environments of Leu8, Ile44, and Val70 are very similar in Ub and di-Ub. To confirm this, we next compared the solvent accessibilities of these and other hydrophobic amino acids in mono- and di-Ub by the use of the water-soluble nitroxide HyTEMPO. This paramagnetic reagent will selectively broaden 1H NMR signals from solvent-exposed residues of a protein (39, 44), and Petros et al. (39) have described the use of HyTEMPO in conjunction with phase-sensitive COSY spectroscopy to distinguish buried from exposed residues in lysozyme and Ub. For Ub, they found that the 1H NMR resonances from Leu8, Ile44, and Val70 were perturbed, whereas signals from hydrophobic residues not on the surface, such as Ile43 and Ile30, were unaffected. We expected that, if the di-Ub crystal structure is retained in solution, 1H-1H cross-peaks from Leu8, Ile44, and Val70 in di-Ub would not be affected by HyTEMPO relative to those in mono-Ub. The HyTEMPO-accessible surface areas of side chains from these and other amino acid residues were calculated based on the mono-, di-, and tetra-Ub crystal structures (Table II). These calculations suggest that the Leu8, Ile44, and Val70 side chains in di-Ub will be protected from the paramagnetic reagent only when di-Ub assumes the crystal structure conformation illustrated in Fig. 3A.

In Fig. 4B, we show that, for monomeric Ub, the intensities of 1H-1H cross-peaks from Leu8, Ile44, and Val70 were reduced by HyTEMPO, whereas signals from the buried residues Ile13 and Ile30 were relatively unaffected (Fig. 4, compare A and B). This result confirms the previous report by Petros et al. (39). We then found that the paramagnetic reagent had essentially identical effects with di- and mono-Ub (Fig. 4, compare A and B with C and D). This suggests that the hydrophobic residues in mono- and di-Ub in solution are similarly accessible to HyTEMPO. As Table II shows, quite different conclusions would be made based on the structures available from x-ray crystallography. Together with the lack of significant chemical shift differences, the results indicate that the structure of di-Ub seen in the protein crystal does not predominate in solution.

Ub(L8A,I44A) Does Not Destabilize a Di-Ub Conformer Critical for Recognition by the PA700 Isopeptidase—Our NMR experiments provide information regarding the average solution

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3 Below 1 mg/ml, neither Ub nor Lys48-linked Ub, oligomers (n ≤ 4) exhibited detectable self-association at pH 6 when examined by analytical ultracentrifugation (R. E. Cohen, unpublished data).
conformation of di-Ub, but do not rule out the possibility that the PA700 isopeptidase specifically recognizes a relatively minor conformer. To address whether the structure represented in the di-Ub crystal is selected by the enzyme, we synthesized a form of di-Ub in which Ub(L8A,I44A) was placed in the proximal position. Our rationale was that, given the 2-fold symmetry of the di-Ub crystal structure (Fig. 3A), destabilization by Ub(L8A,I44A) would be manifested equally in dimers with the Ub variant at either the distal or proximal end. As is shown in Table III, the PA700 isopeptidase clearly can distinguish whether Ub(L8A,I44A) is distal or proximal. Only distal Ub(L8A,I44A) prevents disassembly, whereas the isopeptidase acts equally upon dimers with Ubdiol or Ub(L8A,I44A)diol at the proximal position. Thus, it is unlikely that the di-Ub conformer in the crystal is specifically required by the isopeptidase. Also unlikely is the possibility that distal Ub(L8A,I44A) promotes a new di-Ub conformation that resists cleavage by the isopeptidase; replacement of the surface-exposed Leu8 and Ile44 side chains (Refs. 38 and 39 and also Table II) by methyl groups from alanines would not be expected to stabilize a new Ub-Ub binding interface. Rather, the simplest explanation of the data is that the enzyme interacts directly with Leu8 or Ile44 on the distal Ub of a substrate.

FIG. 4. The hydrophobic side chains of Leu8, Ile44, and Val70 are equally solvent-exposed for Ub and di-Ub in solution. The paramagnetic reagent Hy-TEMPO specifically broadens the NMR signals of surface-exposed hydrophilic residues in Ub, including Leu8, Ile44, and Val70 (39). These perturbations can be detected as diminished 1H-1H cross-peak intensities in a phase-sensitive DQF-COSY NMR spectrum. If Leu8, Ile44, and Val70 side chains are sequestered from solvent in di-Ub, as the crystal structure suggests (see Table II), then these residues should be protected from the effects of the paramagnetic reagent. A and B, DQF-COSY NMR spectra of Ub in 50 mM sodium Pi, pH 6, without and with 20 mM Hy-TEMPO, respectively; C and D, DQF-COSY NMR spectra of di-Ub in 50 mM sodium Pi, pH 6, without and with 20 mM HyTEMPO, respectively. The dashed boxes show positions of the amino acid side chain cross-peaks listed in Table II.
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TABLE II

| Residue | Cross-peak(s) | Accessible side chain surface area
|---------|---------------|-------------------|
|         |               | Mono-Ub | Di-Ub | Tetra-Ub |
| Ile<sup>3</sup> | C<sup>3</sup>H<sup>4</sup>-C<sup>3</sup>H<sup>4</sup> | 0.0 | 0.0 | 0.0 |
| Leu<sup>6</sup> | C<sup>3</sup>H<sup>4</sup>-C<sup>3</sup>H<sup>4</sup> | 11.9 | 0.4 | 9.0 |
| Ile<sup>13</sup> | C<sup>3</sup>H<sup>4</sup>-C<sup>3</sup>H<sup>4</sup>, C<sup>3</sup>H<sup>4</sup>-C<sup>3</sup>H<sup>4</sup> | 0.0 | 0.0 | 2.4 |
| Ile<sup>25</sup> | C<sup>3</sup>H<sup>4</sup>-C<sup>3</sup>H<sup>4</sup>, C<sup>3</sup>H<sup>4</sup>-C<sup>3</sup>H<sup>4</sup> | 2.5 | 3.6 | 3.4 |
| Ile<sup>44</sup> | C<sup>3</sup>H<sup>4</sup>-C<sup>3</sup>H<sup>4</sup> | 2.3 | 0.0 | 3.5 |
| Val<sup>70</sup> | C<sup>3</sup>H<sup>4</sup>-C<sup>3</sup>H<sup>4</sup>, C<sup>3</sup>H<sup>4</sup>-C<sup>3</sup>H<sup>4</sup> | 4.1 | 0.0 | 4.2 |

<sup>a</sup> For these six amino acid residues, only the cross-peaks visible in Fig. 4 are listed.

<sup>b</sup> This was calculated for the portions of the amino acid side chains listed under “Cross-peak(s);” a probe radius of 2.5 Å was used to simulate accessibility to HyTEMPO (39).

<sup>c</sup> Averages of the calculated surface areas for the same residue in each Ub unit are reported.

Kinetic parameters for the disassembly of various Lys<sup>48</sup>-linked diubiquitin substrates by the PA700 isopeptidase

Assays were done as described under “Experimental Procedures.” Each value is the mean ± S.D. calculated from nonlinear fits of the data.

| Diubiquitin substrate | \(K_m\) | \(k_{cat}\) | \(k_{cat}/K_m\) |
|----------------------|----------|-------------|----------------|
| Ub-Ub                | 3.34 ± 0.86 | 4.37 ± 0.42 | 1.31 ± 0.45 |
| Ub-UB(L8A,I44A)diol  | 4.04 ± 0.55 | 5.77 ± 0.94 | 1.18 ± 0.35 |
| Ub(L8A,I44A)-Ubdiol<sup>b</sup> | ND<sup>c</sup> | ND | ND |
| Ub(L8A)-Ubdiol<sup>a</sup> | ND<sup>c</sup> | 0.29<sup>c</sup> | — |
| Ub(I44A)-Ubdiol<sup>a</sup> | 6.73 ± 1.94 | 0.98 ± 0.09 | 0.15 ± 0.06 |
| Ub(K48R)-Ubdiol<sup>a</sup> | ND | ND | ND |
| Ub(K48C)-Ubdiol<sup>a</sup> | ND | ND | ND |
| Ub(K48Aec)-Ubdiol<sup>a</sup> | 2.05 ± 0.67 | 2.69 ± 0.36 | 1.31 ± 0.60 |
| Ub(K48Aec)-Ub<sup>a</sup> | 2.60 ± 1.01 | 2.48 ± 0.43 | 0.95 ± 0.53 |

<sup>a</sup> For these assays, PA700 was increased 10-fold to 80 nM, and up to 20 μM substrate was used.

<sup>b</sup> ND, no products were detected.

<sup>c</sup> —, activity was detected only at the highest substrate concentration employed, and therefore, a \(K_m\) could not be determined.

This value may underestimate \(k_{cat}\) as it is based on a single substrate concentration.

Fig. 5. Inhibition of the PA700 isopeptidase by Ub and Ubdiol.

Disassembly reactions contained the substrate Ub-Ub(L8A,I44A)diol (5.0 μM), PA700 (8 nM), and various amounts of Ub (0–20 μM; circles) or Ubdiol (0–5 μM; squares). Incubation conditions and product analysis by HPLC are described under “Experimental Procedures.” A, the results are shown as a plot of velocity versus [inhibitor]. B, when the data are plotted in the form velocity \(^{-1}\) versus [inhibitor], the ratio of the slopes of the lines indicates that Ubdiol binds to the isopeptidase with approximately seven times the affinity of Ub. The lines drawn in A and B are based on the \(K_m\) values of 5.33 ± 0.88 μM for Ub and 0.76 ± 0.20 μM for Ubdiol, determined from nonlinear fits to the data.

Although structural constraints at the Ub C terminus might allow the PA700 isopeptidase to distinguish proximal Ub from other units in an unanchored poly-Ub chain, distal and internal ubiquitins would nonetheless appear identical by this criterion alone. Why are chains not cleaved internally? Possibly, the conformation of the chain plays a role such that binding of an internal Ub is precluded by steric interference from the adjacent distal Ub. Also, because the Lys<sup>48</sup> side chain is free only in the distal Ub of a Lys<sup>48</sup> linked poly-Ub chain, specific contacts between the enzyme and this lysine side chain could be important in generating the observed distal-end specificity. This possibility was explored by testing as substrates variants of di-Ub synthesized with Ub(K48R), Ub(K48C), or Ub(K48Aec) in the distal position. Whereas no disassembly of Ub(K48C)-Ub dimers was detected, activity was restored either by S-aminooxylation of Cys<sup>48</sup> to form a lysine analog or by the incorporation of Ub(K48R) into the distal position (Table III).

Disassembly of Poly-Ub Chains with Linkages Other than through Lys<sup>48</sup>—Studies of the targeting of poly-Ub conjugates to the 26 S proteasome have focused primarily on chains that
contain Ub–Ub linkages through Lys48. However, in vitro reactions with two different E2 enzymes, mammalian E2<sub>EPF</sub> and yeast Rad6p (encoded by the RAD6/UBC2 gene), yield poly-Ub chains with linkages through Lys11 of Ub or Lys6, respectively (11). Like Lys48-linked chains, these conjugates are recognized by S5a, a poly-Ub-binding protein associated with the 26 S proteasome (11, 45). We have examined whether the PA700 isopeptidase can disassemble these non-Lys48-linked conjugates. Lys11-linked conjugates were generated by the auto-ubiquitination of E2<sub>EPF</sub> in the presence of E1 as described by Baboshina and Haas (11). For Lys6-linked poly-Ub chains, histone H2b was used as the acceptor in a reaction with Rad6p and E1 (11). Ubiquitination reactions were stopped by the addition of EDTA (lanes 1–3) or hexokinase (lanes 4–6) prior to further incubation for 30 min at 37 °C with no additional proteins (lanes 1 and 4), with 74 nM PA700 (lanes 2 and 5), or with 74 nM PA700 and 235 nM Ubal (lanes 3 and 6). In A and B, the compressed bands at the position of the asterisk are artifacts due to the large amount of hexokinase added to the reactions.

Although Lys11 or Lys6 is required for poly-Ub chain formation by these E2 enzymes in vitro, it is possible that other Ub–Ub linkages are incorporated into the conjugates as well.

DISCUSSION

Disassembly of Lys<sub>48</sub>-linked poly-Ub chains by the PA700 isopeptidase was prevented when Ub in the substrate was replaced by Ub(L8A,I44A). Although the crystal structure of di-Ub highlighted Leu<sup>8</sup> and Ile<sup>44</sup> for their role in stabilizing a compact folded conformation of the covalent Ub-Ub dimer (22), the results of our <sup>1</sup>H NMR experiments show that this structure does not represent the predominant conformation of di-Ub in solution. Moreover, the 2-fold symmetry of di-Ub in the crystal is incompatible with the distinct effects on isopeptidase activity observed when Ub(L8A,I44A) was incorporated into the distal versus proximal position of di-Ub substrates. We conclude that Leu<sup>8</sup> and Ile<sup>44</sup> most likely interact directly with the PA700 isopeptidase and that this interaction is restricted to the distal Ub. Substrates with single amino acid substitutions in Ub showed that Leu<sup>8</sup> is more critical than Ile<sup>44</sup> for enzyme activity.

From our NMR data, we cannot evaluate whether di-Ub in solution adopts the conformation seen in the tetra-Ub crystal structure (Fig. 3B). However, because each Ub unit of tetra-Ub in the crystal makes multiple contacts with both its proximal and distal neighbors (40), the “tetra-Ub conformation” is not likely to be populated significantly by di-Ub. Even with longer Lys<sub>48</sub>-linked poly-Ub chains that might adopt the tetra-Ub conformation, the distal-end Ub still would have side chains from Leu<sup>6</sup> Ile<sup>44</sup>, and Lys<sup>48</sup> exposed at its surface. Thus, we expect that Lys<sub>48</sub>-linked poly-Ub chains of any length will display the substrate determinants recognized by the PA700 isopeptidase.

Despite the minor role that the di-Ub crystal conformation appears to play for di-Ub in solution, the <sup>1</sup>H NMR chemical shift data in Table I hint that the structure determined crystallographically does contribute to the average chain conformation. Transient associations between the hydrophobic patches on each Ub unit of di-Ub are likely to be responsible for the small upfield shifts of the Leu<sup>6</sup> and Ile<sup>44</sup> side chain resonances observed in di-Ub versus mono-Ub. Presumably, these associations help to stabilize the Ub-Ub dimer interface (22). Although not specifically recognized by the PA700 isopeptidase, a di-Ub “cap” structure as depicted in Fig. 3A could be important for interactions of other Ub system proteins with the end(s) of Lys<sub>48</sub>-linked poly-Ub.

Beal et al. (21) had reported that polyubiquitinated α-lactalbumin conjugates assembled with Ub(L8A,I44A) resist degradation. This was the first indication that Ub residues Leu<sup>8</sup> and Ile<sup>44</sup> are important for Ub-dependent proteolysis. Originally,
the primary cause for this effect was thought to be the inability of poly-Ub(L8A,I44A) to bind to the S5a subunit of the 26 S proteasome. That the yeast homolog of S5a is dispensable in vitro (Refs. 50 and 51 and see below) implicates Ub residues Leu and Ile in the recognition of conjugates by other poly-Ub-binding proteins on the proteasome. Thus, the same structural features of poly-Ub can be used by multiple components of the Ub-proteasome degradation system. Further evidence of this is provided by our demonstration that Ub(L8A,I44A) can have a profound effect upon the PA700 isopeptidase. Mutant forms of Ub have been employed widely to probe structure-function relationships in ubiquitination and Ub-dependent proteolysis (e.g. Refs. 9 and 21); our results show that in such studies the potential effects of altered Ub structure upon deubiquitination also must be considered. We have shown previously that the isopeptidase and S5a are distinct subunits of the PA700 regulatory complex (20). It is surprising that the activities of both proteins involve Leu and Ile in Ub, and both recognize Lys-6-, Lys-11-, and Lys-48-linked poly-Ub. These common properties raise the possibility that S5a may help to position substrates for processing by the isopeptidase. Although at present we cannot disprove this idea, it seems unlikely. The relatively low affinity of S5a for Ub and short poly-Ub chains (21, 45) contrasts strongly with the behavior we observe for the isopeptidase. Not only do Ub and di-Ub appear to have similar affinities for the isopeptidase (and Km values of 10-5 to 10-6 M), but deubiquitination reactions with various Ub-protein conjugates (n = 1–4) have similar rates and are not processive (52). A direct role for S5a in substrate presentation to the PA700 isopeptidase is difficult to reconcile with these results.

Despite the preference of S5a for binding poly-Ub chains of n > 4 in vitro (45), poorly ubiquitinated conjugates still can be degraded by the 26 S proteasome (6, 7, 20). Thus, either the chain-length specificity of S5a is less stringent when it is associated with the 26 S complex, or other Ub-poly-Ub-binding proteins also reside in the complex. This latter possibility is supported by the recent reports that yeast mutated to lack the PA700 isopeptidase (53). Similarly, the PA700 isopeptidase may function to rescue proteins before they are degraded by the 26 S proteasome. Evidence to support this idea has been presented (20).

The Km of 5 × 10^-5 M that we have determined for the PA700 isopeptidase with Ub approximates the intracellular concentrations reported for free Ub (58, 59). Assuming that there is no compartmentalization of PA700 and 26 S proteasomes from this pool, competition with free Ub would be expected to decrease significantly the PA700 isopeptidase activity; ~10^-5 M Ub would double the apparent Km of the isopeptidase for Ub-protein or poly-Ub-protein conjugates. Thus, fluctuations in cellular Ub levels such as in response to heat shock or other stresses (60) could modulate the activity of the PA700 isopeptidase.

In vitro, the PA700 isopeptidase was shown to bias protein degradation by the 26 S proteasome toward polyubiquitinated proteins (20). Low Ub concentration would increase deubiquitination by the isopeptidase, which in turn would increase the stringency of this selection for the most highly ubiquitinated substrates. Whether poly-Ub disassembly by the PA700 isopeptidase serves an additional role in the degradation of polyubiquitinated substrates remains to be determined.

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REFERENCES
1. Ciechanover, A. (1994) Cell 79, 13–21
2. Hochstrasser, M. (1996) Annu. Rev. Genet. 30, 405–439
3. Herschko, A., and Heller, H. (1985) Biochem. Biophys. Res. Commun. 128, 1079–1086
4. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) Science 243, 1576–1580
5. Finley, D., Sadis, S., Menia, B. P., Boucher, P., Ecker, D. J., Crooke, S. T., and Chau, V. (1994) Mol. Cell. Biol. 14, 5501–5509
6. Haas, A. L., Rebeck, P. M., Pratt, G., and Rechtesteiner, M. (1990) J. Biol. Chem. 265, 21664–21669

We presume that Ubal inhibits the Doa4p and PA700 isopeptidases competitively, although thus far, experimental proof has been precluded by the tight association of Ubal with these enzymes.

5 E. Swain and R. E. Cohen, unpublished data. 6 We presume that Ubal inhibits the Doa4p and PA700 isopeptidases competitively, although thus far, experimental proof has been precluded by the tight association of Ubal with these enzymes.
