A Ubiquitin C-terminal Isopeptidase That Acts on Polyubiquitin Chains

ROLE IN PROTEIN DEGRADATION*

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In the ubiquitin (Ub) pathway, proteins are ligated with polyUb chains and then are degraded by a 26 S protease complex. We describe an enzyme, called isopeptidase T, that acts on polyUb chains. It is a monomeric Ub-binding protein abundant in erythrocytes and reticulocytes. The activity of the isopeptidase is inhibited by iodoacetamide and Ub aldehyde. Treatment of the enzyme with Ub aldehyde increased its affinity for free Ub, indicating the existence of two different Ub-binding sites and cooperativity between the two sites.

Isopeptidase T acts on polyUb-protein conjugates, but not on conjugates in which the formation of polyUb chains was prevented by the use of reductively methylated Ub or on abnormal polyUb chains formed with a mutant Ub that contains a Lys → Arg substitution at residue 48. The enzyme converts high molecular mass polyUb-protein conjugates to lower molecular mass forms with the release of free Ub, but not of free protein substrate. The lower molecular mass Ub-protein conjugate products are resistant to further action of the enzyme. Isopeptidase T stimulates protein degradation in a system reconstituted from purified enzyme components. The enzyme also stimulates the degradation of proteins ligated to polyUb chains by the 26 S protease complex. Preincubation of polyUb-protein conjugates with the isopeptidase did not much increase their susceptibility to proteolysis by the 26 S complex. On the other hand, preincubation of conjugates with the 26 S protease complex and ATP increased the release of free Ub upon further incubation with the isopeptidase. It thus seems that a role of this isopeptidase in protein breakdown is to remove polyUb chain remnants following the degradation of the protein substrate moiety by the 26 S complex.

In the ubiquitin (Ub) pathway, proteins are marked for degradation by covalent ligation to this polypeptide process, several Ub molecules are linked via their C termini to ε-amino groups of Lys residues of the protein by isopeptide linkages. In addition, polyUb chains are bound to the protein substrate (1), in which each additional Ub unit is usually bound to Lys⁴⁸ of the previous Ub (2). Proteins ligated to multiple units of Ub are degraded by an ATP-dependent 26 S protease complex (reviewed in Refs. 3–5).

Essential to the operation of the proteolytic system is the recycling of Ub. Three types of Ub C-terminal isopeptidase activities may be expected to be involved in protein breakdown: (a) an isopeptidase activity is required for the release of Ub that is linked to the Lys residues of the protein substrate during or following the proteolytic degradation of the substrate; (b) isopeptidase activity would be expected to disassemble polyUb chains during or after proteolysis; and (c) an isopeptidase activity that releases undegraded protein from Ub-protein conjugates may play a correction role in salvaging inappropriately ubiquitinated proteins (6). Several Ub C-terminal isopeptidases and hydrolases have been described (7–17), but their possible involvement in the above processes has not been defined.

Part of the difficulty in determining the relevance of a Ub C-terminal hydrolase or isopeptidase to protein breakdown is due to the possibility that some of these enzymes may have nonproteolytic functions. Monoubiquitination of some proteins may have a role in their modification, and the function of the corresponding isopeptidase may be to reverse the modification. In addition, some Ub C-terminal α-NH₂-protein hydrolases are certainly involved in the processing of biosynthetic precursors of Ub since Ub genes either are arranged in head-to-tail polyUb arrays or are fused to ribosomal proteins (18–20). One way to determine the involvement of an isopeptidase in protein breakdown is by showing that it is required for the activity of a reconstituted proteolytic system. In this study, we describe an isopeptidase that acts on polyUb structures and show that it stimulates the proteolysis of proteins ligated to polyUb chains.

EXPERIMENTAL PROCEDURES

Materials—Ub from bovine erythrocytes, horse heart cytochrome c, HoLA, and reduced and carboxymethylated bovine serum albumin were obtained from Sigma, and yeast hexokinase (140 units/mg) was from Boehringer Mannheim. All proteins were radioiodinated by the chloramine-T procedure as described (21). Molecular mass marker proteins were from Sigma, and prestained molecular mass markers were from Bio-Rad. MeUb (1) and Ubal (9) were prepared as described previously. Samples of UbrR48 (2) were generously provided by Drs. V. Chau and V. Fried; identical results were obtained with both samples.

Preparation of Enzymes.—Fraction II (proteins bound to DEAE-cellulose and eluted with 0.5 M KCl) was prepared from human
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earthrocytes as described previously (22). It was subjected to affinity chromatography on Ub-Sepharose in the absence of ATP by a procedure similar to that described previously for the purification of E₃ from reticulocytes (23). The pH 9 eluate of this affinity procedure served as the source of isopeptidase for this study. The purification of the isopeptidase is described under "Results." Unless otherwise stated, the purified preparation following the FPLC step (2.5 pmol/μl) was used. When specified, we used the partially purified isopeptidase following the Ultrogel step (2.5 pmol/μl). The amount of enzyme was determined by its binding to ¹²⁵I-Ub as described below.

 pepidase, whereas the rest was free ¹²⁵I-HaLA. Free ¹²⁵I-HaLA cannot be separated from ¹²⁵I-HaLA conjugates by gel filtration because HaLA is present at a high molecular mass aggregate under the presently used reaction conditions.

Assay of Free Ub—The release of free Ub from conjugates was estimated by an end-point assay that measures the formation of [³H]AMP-Ub with E₁ (28). Prior to the assay, unlabeled ATP was removed from samples by repeated precipitations with 12% trichloroacetic acid as described (29), except that following the second precipitation, the sediment was dissolved with 90 μl of 0.1 N NaOH and then neutralized with 1 N HCl in the presence of 10 mM Tris. A sample of 50 μl was mixed with 50 μM Tris-HCl (pH 7.6), 5 mM MgCl₂, 10 pmol of [²⁸³H]AMP (ICN; 30 Ci/mmol), and 2 pmol of iodacetamide-treated E₁ in a final volume of 70 μl. Following incubation at 37°C for 10 min, [²⁸³H]AMP-Ub was precipitated with 12% trichloroacetic acid, and its radioactivity was estimated as described (29). In samples containing ¹²⁵I-labeled proteins, the precipitate was dissolved with 50 μl of 0.1 N NaOH and reprecipitated with 450 μl of 12% trichloroacetic acid, and radioactivity of [²⁸³H]AMP released to the supernatant was estimated by scintillation counting.

RESULTS

Purification of Ub-binding Protein and Identification of Its Isopeptidase Activity—The initial objective of this study was to identify the functions of an abundant Ub-binding protein previously observed in eluates of Ub-Sepharose affinity columns. In this affinity purification procedure, E₁ and E₂ enzymes are eluted from the Ub affinity column under specific conditions, and all other Ub-binding proteins are eluted together by raising the pH to 9.0 (24). The most abundant protein in pH 9 eluates of Ub-Sepharose affinity chromatography of extracts of reticulocytes and erythrocytes has a subunit size of 100 kDa (24, 30). This protein was further purified from Ub affinity eluates of erythrocytes by gel filtration chromatography on Ultrogel ACA-34. Purification was followed by SDS-polyacrylamide gel electrophoresis (Fig. 1A) and by a quantitative assay for the binding of ¹²⁵I-Ub to proteins (Fig. 1B). The 100-kDa band eluted in coincidence with Ub binding activity at an apparent native molecular size of 100 kDa (Fig. 1, A and B). Thus, the protein is apparently composed of a single subunit. The gel filtration procedure separates the major Ub-binding protein from a 50-kDa Ub C-terminal hydrolase (7, 8), but several minor protein bands in the molecular mass region of 50–95 kDa are eluted in coincidence with the major 100-kDa band (Fig. 1A). On further purification by anion-exchange chromatography on an FPLC Mono Q column, the major Ub-binding protein was eluted in a sharp peak at ~350 mM NaCl, but most of the lower molecular mass minor protein bands coeluted again with the major 100-kDa band (Fig. 2A). The lower molecular mass proteins could not be separated from the 100-kDa band by other purification procedures, such as gradient elution on hydroxyapatite and hydrophobic chromatography on phenyl-Sepharose (data not shown). It thus seemed either that the lower molecular mass minor proteins are tightly associated with the 100-kDa protein or that they are products of partial proteolysis of the 100-kDa subunit that remained associated under nondenaturing conditions. To examine these possibilities, antibodies were raised against the pure 100-kDa subunit following its excision from SDS-polyacrylamide gels. When tested by immunoblotting, this antisem reacted not only with the 100-kDa band, but...
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Fig. 1. Gel filtration chromatography of ubiquitin-binding protein from human erythrocytes. 1.0 ml of the pH 9 eluate of Ub-Sepharose affinity chromatography of extract of human erythrocytes (containing ~3.5 mg of protein) was applied to a column (0.9 × 56 cm) of Ultrogel AcA-34 (Pharmacia LKB Biotechnology Inc.) equilibrated with 50 mM Tris-HCl (pH 7.2) and 1 mM DTT. Fractions of 0.93 ml were collected at a flow rate of 0.93 ml per hour. Samples (15 μl) of column fractions were subjected to electrophoresis on SDS-10% polyacrylamide gels and stained with Coomassie Blue. Numbers to the left indicate the positions of molecular mass markers (in kilodaltons), and fraction numbers are indicated at the top. The arrow to the right indicates the position of ovalbumin, 0.15 pg of ovalbumin, 0.15 pg of cytochrome C, and 2 pg of cytochrome C3, respectively. 1.0 ml of the pH 9 eluate of Ub-Sepharose affinity chromatography of extract of human erythrocytes was applied to a column (0.9 × 56 cm) of Ultrogel AcA-34 (Pharmacia LKB Biotechnology Inc.) equilibrated with 50 mM Tris-HCl (pH 7.2) and 1 mM DTT. Fractions of 0.93 ml were collected at a flow rate of 0.93 ml per hour. Samples (15 μl) of column fractions were subjected to electrophoresis on SDS-10% polyacrylamide gels and stained with Coomassie Blue. Numbers to the left indicate the positions of prestained molecular mass markers (in kilodaltons), and fraction numbers are indicated at the top.

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also with all the lower molecular mass minor bands (data not shown). It thus seems that the lower molecular mass bands are partial proteolysis fragments of the 100-kDa band.

We next examined whether the function of the 100-kDa protein is related to that of the 30-kDa Ub C-terminal hydrolase. The latter enzyme preferentially cleaves the linkage of Ub to small compounds (8). Adducts of Ub to small molecules (such as Ub-CONH₂) that are good substrates for the 30-kDa hydrolase were cleaved by the 100-kDa enzyme at a relatively low rate (data not shown). On the other hand, the enzyme rapidly degrades high molecular mass Ub-protein conjugates. The disassembly of high molecular mass conjugates was accompanied by the release of free Ub. That this isopeptidase activity indeed belongs to the 100-kDa Ub-binding protein was examined by coincidence in purification. As shown in Fig. 1C, enzyme activity that disassembles high molecular mass conjugates coincided exactly with the Ub-binding protein in fractions of the gel filtration column (peak center at fraction 27). Similarly, isopeptidase activity eluted in coincidence with the 100-kDa band on FPLC separation on a Mono Q column (Fig. 2B, peak center at fraction 13). It thus seems that this Ub-binding protein has isopeptidase activity. To distinguish it from the many other Ub C-terminal isopeptidases and hydrolases that have been described in eukaryotic cells (7–17), we propose to call it isopeptidase T.

Characterization of Isopeptidase Action—High molecular mass Ub-protein conjugates contain at least two types of isopeptidase linkages. Some Ub molecules are linked to amino groups of the substrate protein. In addition, such Ub-protein conjugates usually contain polyUb chains (1) in which a major site of the linkage is at Lys48 of Ub (2). To examine which type of linkage is subject to the action of isopeptidase T, conjugates of 125I-HoLA with different derivatives of Ub were prepared. These included a derivative of Ub in which amino groups were blocked by reductive methylation (MeUb) (1) and a mutant Ub in which the Lys residue at position 48 has been replaced by Arg (UbR48) (2). MeUb is efficiently ligated to proteins, but cannot form polyUb chains (1), whereas with UbR48, the formation of polyUb linkages at Lys48 is specifically prevented (2). As shown in Fig. 3, with native Ub, high molecular mass (>150 kDa)polyUb conjugates of HoLA are formed. With MeUb, 10–12 conjugates of HoLA are produced, the largest of which has an apparent molecular mass of ~120 kDa. This is consistent with the presence of HoLA of 12 Lys residues. The pattern of conjugates produced with UbR48 resembled that of Ub conjugates (rather than that of MeUb conjugates), except that the proportion of high molecular mass conjugates was decreased relative to low molecular mass conjugates. This indicates that some polyUb chain formation can take place in the absence of Lys48 of Ub. To examine the action of isopeptidase T on the different types of isopeptide linkages, the above derivatives were incubated with the enzyme, the reaction products were separated by gel electrophoresis (Fig. 3), and radioactivity at different regions of the gel was quantified (Table 1). Incubation of conjugates of native Ub with isopeptidase T converted high molecular mass (>150 kDa) to medium-sized (50–150 kDa) derivatives. By contrast, there was little, if any effect of isopeptidase T on conjugates of MeUb or UbR48 (Fig. 3 and Table 1). These findings suggest that isopeptidase T acts preferentially on Ub-Lys48.
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The column described for Fig. 1 (which contained 1800 pmol of Ub-binding protein) was applied to an analytical Mono Q HR 5/10 column (Pharmacia LKB Biotechnology Inc.) equilibrated with 50 mM Tris-HCl (pH 7.6) and 1 mM DTT. The column was eluted with a 0-500 mM KCl linear gradient, with a slope of 10 mM/min, at a flow rate of 1 ml/min. Fractions of 1 ml were collected, starting from 20 min. The fractions were collected into chilled tubes that contained 0.5 mg of ovalbumin. Salt was removed by repeated dilution (>100-fold) with 20 mM Tris-HCl (pH 7.6) and 1 mM DTT. The column was eluted with a 0-500 mM KCl linear gradient, with a slope of 10 mM/min, at a flow rate of 1 ml/min. Fractions of 1 ml were collected, starting from 20 min. The fractions were collected into chilled tubes that contained 0.5 mg of ovalbumin. Salt was removed by repeated dilution (>100-fold) with 20 mM Tris-HCl (pH 7.6) containing 1 mM DTT and concentration to 0.5 ml in CF-25 ultrafiltration cones (Amicon Corp.), polyacrylamide gel electrophoresis. Samples (15 μl) of column fractions were separated by electrophoresis on SDS-10% polyacrylamide gels and visualized by silver staining (Bio-Rad). Numbers to the left indicate the positions of molecular mass markers (in kilodaltons), and numbers at the top are fraction numbers. Ov, carrier ovalbumin. The arrow marks the position of the 100-kDa protein. B, assay of isopeptidase activity and stimulation of proteolysis of 125I-HoLA-Ub conjugates. Δ, isopeptidase activity. 125I-Ub-HoLA conjugates were prepared and purified on Sephadex G-100 as described under "Experimental Procedures." 125I-Ub-HoLA conjugates (containing 7.5 pmol of Ub) were incubated with 0.05 μl of column fractions in the presence of 20 mM Tris-HCl (pH 7.6), 1 mM DTT, and 20 μg of bovine serum albumin in a final volume of 30 μl. Following incubation at 37 °C for 60 min, the liberation of free Ub was determined as described under "Experimental Procedures." O, proteolysis of conjugates. Samples (0.2 μl) of column fractions were incubated with 5 μl of purified 26 S complex and 125I-Ub conjugates, and the release of acid-soluble radioactivity was determined under the conditions described under "Experimental Procedures." Conjugate breakdown without added isopeptidase (3.5%) was subtracted from the results.

Ub linkages in polyUb chains. This conclusion is consistent with a recent report of Chen and Pickart (31), who noted that a 100-kDa isopeptidase acts on Lys48 linkages in polyUb chains formed by a certain species of Ub carrier protein.

![Graph](image)

**Fig. 2. Purification of isopeptidase by anion-exchange FPLC.** A sample (0.3 ml) of the pooled peak from the Ultrogel column described for Fig. 1 (which contained 1800 pmol of Ub-binding protein) was applied to an analytical Mono Q HR 5/10 column (Pharmacia LKB Biotechnology Inc.) equilibrated with 50 mM Tris-HCl (pH 7.6) and 1 mM DTT. The column was eluted with a 0-500 mM KCl linear gradient, with a slope of 10 mM/min, at a flow rate of 1 ml/min. Fractions of 1 ml were collected, starting from 20 min. The fractions were collected into chilled tubes that contained 0.5 mg of ovalbumin. Salt was removed by repeated dilution (>100-fold) with 20 mM Tris-HCl (pH 7.6) containing 1 mM DTT and concentration to 0.5 ml in CF-25 ultrafiltration cones (Amicon Corp.). A, polyacrylamide gel electrophoresis. Samples (15 μl) of column fractions were incubated with 0.05 μl of column fractions in the presence of 20 mM Tris-HCl (pH 7.6), 1 mM DTT, and 20 μg of bovine serum albumin in a final volume of 30 μl. Following incubation at 37 °C for 60 min, the liberation of free Ub was determined as described under "Experimental Procedures." O, proteolysis of conjugates. Samples (0.2 μl) of column fractions were incubated with 5 μl of purified 26 S complex and 125I-Ub conjugates, and the release of acid-soluble radioactivity was determined under the conditions described under "Experimental Procedures." Conjugate breakdown without added isopeptidase (3.5%) was subtracted from the results.

**Fig. 3. Isopeptidase T acts preferentially on conjugates with polyUb chains containing Ub-Lys48-Ub linkages.** Different conjugates of 125I-HoLA were prepared as described for 125I-HoLA-Ub conjugates (see "Experimental Procedures"), except that where indicated, Ub was replaced by MeUb or UbR48. Samples (12 μl) of conjugate preparations were incubated with (+) or without (−) 1 μl of isopeptidase under conditions identical to those described for conjugate proteolysis (see "Experimental Procedures"), except that the 26 S complex was omitted. Following incubation at 37 °C for 60 min, the samples were separated on an SDS-10% polyacrylamide gel.

**TABLE I**

| Conjugate size | Change due to isopeptidase |
|----------------|---------------------------|
| kDa            | Ub | MeUb | UbR48 |
| >150           | -6.0 (17.5) | +0.3 (39.6) | +0.3 (9.1) |
| 50-150         | +3.2 (11.6) | +0.3 (7.9) | +0.3 (5.9) |
| 20-50          | +2.5 (4.9) | +0.3 (7.9) | +0.3 (5.9) |

It is interesting to note that some high molecular mass and medium-sized conjugates of Ub are resistant to the action of isopeptidase T. In the experiments summarized in Fig. 4, 125I-HoLA-Ub conjugates were incubated with increasing concentrations of isopeptidase T; and following gel electrophoresis, the levels of conjugates of different sizes and of free 125I-HoLA were quantified. The increase in levels of high molecular mass conjugates was accompanied by an increase in levels of medium-sized conjugates. There was a small increase in levels of low molecular mass conjugates and no significant increase in free 125I-HoLA. Even at high concentrations of isopeptidase T, most medium-sized conjugates (and about one-half of high molecular mass conjugates) were not degraded further. These results suggest that the action of isopeptidase T on high molecular mass conjugates may be limited by the availability of susceptible isopeptide linkages (see "Discussion"). From the absence of liberation of free 125I-HoLA, it is concluded that this enzyme does not cleave linkages between Ub and the substrate protein.

Like most other Ub C-terminal hydrolases, the activity of isopeptidase T is inhibited by iodoacetamide and Ub aldehyde.
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Isopeptidase T Stimulates Protein Degradation—We next asked whether isopeptidase T has a role in protein breakdown. This could be tested in a proteolytic system reconstituted from purified components from reticulocytes. Since Ub-protein conjugates are intermediates in protein degradation, inhibition of protein breakdown by the isopeptidase could be expected. Surprisingly, however, a marked stimulation of protein degradation by isopeptidase T was observed (Fig. 6A). Under the standard conditions of assay, the rate of protein breakdown was increased 2–3-fold by the isopeptidase. The extent of the stimulation of protein breakdown by isopeptidase T depended upon the concentration of two of the components of the reconstituted proteolytic system. The rate of protein breakdown was inhibited by high concentrations of E₆, and at least a part of this inhibition was abolished by the supplementation of isopeptidase T (Fig. 6B). An opposite effect was observed when the concentration of the 26 S protease complex was varied; the extent of the stimulation of protein degradation was much greater at low concentrations of the 26 S complex than at high concentrations (Fig. 6C).

To examine whether the stimulation of protein degradation was indeed caused by isopeptidase T (or by a contaminating enzyme in the preparation used), we tested whether the stimulatory activity coincides with the 100-kDa subunit in enzyme purification. A good coincidence was found in fractions of gel filtration chromatography (Fig. 1B) and in separation on a Mono Q column (data not shown). Most of the experiments on the stimulation of protein breakdown by isopeptidase T were carried out with 125I-HoLA substrate. However, similar

Fig. 4. Influence of increasing concentrations of isopeptidase T on breakdown of high molecular mass poly Ub-protein conjugates. 125I-HoLA-Ub conjugates were incubated with the indicated amounts of the isopeptidase under the conditions described for Fig. 3. Following incubation for 37 °C for 60 min, the samples were separated by electrophoresis on SDS-10% polyacrylamide gels and autoradiographed, and the indicated regions were quantified by γ-counting. The results are expressed as the percentage of total radioactivity in each lane.

Fig. 5. Influence of ubiquitin aldehyde on release of 125I-Ub from isopeptidase. The isopeptidase (3 pmol) was incubated in 25 μl of a buffer consisting of 50 mM Tris-HCl (pH 7.2), 1 mM DTT, and 2 mg/ml cytochrome c in the presence (O) or absence (△) of 40 pmol of Ub. Following incubation at 37 °C for 10 min, 3 pmol of 125I-Ub was added in 25 μl of the above buffer, and incubation was continued for a further 15 min at 37 °C. The mixture was then transferred to 20 °C, and the release of 125I-Ub from the enzyme was initiated by the supplementation of 1290 pmol of unlabeled Ub. At various times afterwards, the amount of 125I-Ub bound to the enzyme was estimated by binding to DEAE-cellulose as described under “Experimental Procedures,” except that adsorption to resin and washes were performed rapidly at 0 °C. All results were corrected for the amount of 125I-Ub bound nonspecifically to DEAE-cellulose as estimated by bound radioactivity that remained following a 25-min period of exchange with unlabeled Ub.
The degradation of 125I-HoLA in the reconstituted proteolytic system was estimated as described under "Experimental Procedures," following incubation for the time periods indicated, in the presence (●, +IP) or absence (○, Control) of 1 μl of isopeptidase following the Ultrogel purification step. B, influence of E2 concentration. Experimental conditions were as described for A, except that the concentration of E2 was varied as indicated, and incubation was carried out for 60 min. C, influence of concentration of 26 S complex. Experimental conditions were as described for A, except that the concentration of the 26 S complex (purified on Sepharose 6B) was varied as indicated, and incubation was carried out for 60 min.

FIG. 6. Stimulation of protein breakdown by isopeptidase T in reconstituted system from reticulocytes. A, time course. The degradation of 125I-HoLA in the reconstituted proteolytic system was estimated as described under “Experimental Procedures,” following incubation for the time periods indicated, in the presence (●, +IP) or absence (○, Control) of 1 μl of isopeptidase following the Ultrogel purification step. B, influence of E2 concentration. C, influence of concentration of 26 S complex. Experimental conditions were as described for A, except that the concentration of the 26 S complex (purified on Sepharose 6B) was varied as indicated, and incubation was carried out for 60 min.

FIG. 7. Synergistic effect of isopeptidase T and 26 S protease complex on conjugate breakdown in presence of ATP. 125I-HoLA-Ub conjugates were formed as described under “Experimental Procedures.” The reaction mixture contained, in a volume of 50 μl, 40 mM Tris-HCl (pH 7.6), 4 mM MgCl2, 2.5 mM DTT, 75 μg of ovalbumin, and 10 μl of 125I-HoLA-Ub conjugates (~290,000 cpm). Where indicated, 5 μl of 26 S protease complex or 1 μl of isopeptidase was added. Incubation was carried out with 2 mM ATP (+ATP) or without ATP and in the presence of 120 μg/ml hexokinase and 10 mM 2-deoxyglucose (−ATP). Following incubation at 37°C for 60 min, samples were separated on an SDS-10% polyacrylamide gel.

Effects were obtained with other protein substrates, such as lysozyme, oxidized ribonuclease, or β-lactoglobulin (data not shown).

Isopeptidase T Stimulates Degradation of Ub-conjugated Protein by 26 S Protease Complex—The observation that the extent of the stimulation of protein degradation by isopeptidase T is greater when the system is rate-limited by the 26 S protease complex (Fig. 6C) suggested that the isopeptidase may stimulate the activity of the protease complex. This possibility was examined in the experiment shown in Fig. 7, in which the action of the 26 S complex or isopeptidase T or their combination on the breakdown of 125I-HoLA-Ub conjugates was tested in the presence or absence of ATP. ATP is required for the activity of the 26 S complex (27, 33). By contrast, the action of the isopeptidase to convert high molecular mass to medium-sized conjugates is not influenced by ATP (Fig. 7). Therefore, ATP dependence indicates the involvement of protease function. When 125I-HoLA-Ub conjugates were incubated with both the 26 S protease and isopeptidase in the absence of ATP, conjugate breakdown was not much greater than that obtained by incubation with the isopeptidase alone. On the other hand, in the presence of ATP, incubation with both the protease and isopeptidase caused a pronounced degradation of both high molecular mass and medium-sized conjugates. Incubation with the 26 S protease alone in the presence of ATP caused partial degradation of high molecular mass conjugates. However, conjugate breakdown with both enzymes under these conditions was markedly synergistic, i.e. the extent of degradation obtained by incubation with both the protease and isopeptidase was greater than the sum of the effects of incubations with one of these enzymes. This suggests an interdependence of the activities of the 26 S protease and isopeptidase. Conjugate breakdown as seen in Fig. 7 could be due either to disassembly by the isopeptidase or to proteolysis by the protease. Therefore, possible explanations for the synergistic effect are that isopeptidase action stimulates the 26 S protease, that protease action stimulates that isopeptidase, or that both processes take place.

The possible stimulation of protease action by the isopeptidase was examined by following the proteolysis of 125I-HoLA-Ub conjugates to acid-soluble products. As shown in Fig. 8A, the isopeptidase stimulated conjugate proteolysis by the 26 S protease complex. Control experiments showed that the isopeptidase preparations used had no protease activity. Proteolysis of Ub-conjugated HoLA was stimulated by low concentrations of the isopeptidase (Fig. 8B), similar to those required for the stimulation of breakdown of free HoLA in the reconstituted system. The magnitude of the stimulation of conjugate proteolysis varied between 1.5- and 3-fold with different preparations of 125I-HoLA-Ub conjugates. The stimulation of conjugate proteolysis by the isopeptidase is specific to conjugates that contain polyUb chains; the proteolysis of 125I-lysozyme-MeUb conjugates, which are good substrates for the 26 S protease (27), is not affected by isopeptidase T (data not shown).

Relationship between Actions of Isopeptidase and Protease Complex—One possible explanation for the stimulatory effect
of the isopeptidase on conjugate breakdown is that the isopeptidase converts polyUb-protein conjugates to a form more susceptible to degradation by the 26 S proteasome. For example, excessively long polyUb chains may be "trimmed" by the isopeptidase to derivatives preferred by the protease. This possibility was tested by the two-stage experiment shown in Fig. 9. In the first stage, 125I-HoLA-Ub conjugates were incubated with or without isopeptidase, and then the preparations were treated with 0.2 N NaOH to inactivate the enzyme. In the second stage, these preparations of conjugates were incubated with the 26 S protease in the presence or absence of fresh isopeptidase. If high molecular mass conjugates are converted by the isopeptidase to forms susceptible to the action of the 26 S protease, it is to be expected that following treatment with the isopeptidase, such conjugates would be degraded at a high rate in the control incubation with the 26 S protease only and that added fresh isopeptidase would not stimulate further rates of proteolysis. As seen in Fig. 9, pretreatment of conjugates with the isopeptidase slightly stimulated rates of proteolysis, but the magnitude of further stimulation with fresh isopeptidase was essentially unaffected by prior treatment with the isopeptidase. Control experiments showed that pretreatment with the isopeptidase produced essentially maximal conversion of high molecular mass to medium-sized conjugates and that the isopeptidase was completely inactivated after the first stage by treatment with alkali. These results indicate that most of the stimulatory effect of the isopeptidase on conjugate proteolysis is not due to the conversion of conjugates to forms susceptible to degradation by the protease complex.

We next asked whether interaction between the protease and isopeptidase influences the release of free Ub from conjugates. In the experiment shown in Table II, conjugates were incubated with the protease or isopeptidase or both, and the release of free Ub was determined by a sensitive enzymatic assay. In the presence of ATP, the release of free Ub promoted by the combination of the protease and isopeptidase greatly exceeded the sum of Ub release obtained by incubations with each enzyme alone. This synergistic effect suggested that the ATP-dependent action of the protease may convert conjugates to derivatives susceptible to isopeptidase activity. This possibility was tested by the two-stage experiment shown in Fig. 9. In the first stage, 125I-HoLA-Ub conjugates were incubated with or without isopeptidase, and then the preparations were treated with 0.2 N NaOH to inactivate the enzyme. In the second stage, these preparations of conjugates were incubated with the 26 S protease in the presence or absence of fresh isopeptidase. If high molecular mass conjugates are converted by the isopeptidase to forms susceptible to the action of the 26 S protease, it is to be expected that following treatment with the isopeptidase, such conjugates would be degraded at a high rate in the control incubation with the 26 S protease only and that added fresh isopeptidase would not stimulate further rates of proteolysis. As seen in Fig. 9, pretreatment of conjugates with the isopeptidase slightly stimulated rates of proteolysis, but the magnitude of further stimulation with fresh isopeptidase was essentially unaffected by prior treatment with the isopeptidase. Control experiments showed that pretreatment with the isopeptidase produced essentially maximal conversion of high molecular mass to medium-sized conjugates and that the isopeptidase was completely inactivated after the first stage by treatment with alkali. These results indicate that most of the stimulatory effect of the isopeptidase on conjugate proteolysis is not due to the conversion of conjugates to forms susceptible to degradation by the protease complex.

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enzyme was inactivated by treatment with acid. In the second stage, reaction products were incubated in the presence or absence of the isopeptidase, and the final products were analyzed by quantitative polyacrylamide gel electrophoresis. It may be seen from Table III that following the first incubation (no isopeptidase added in the second stage), treatment with the 26 S protease in the presence of ATP converted a portion of the high molecular mass (＞150 kDa) 125I-Ub-HaLA conjugates to low molecular mass and medium-sized (20-150 kDa) derivatives. This observation, made with conjugates labeled with 125I-Ub, was different from previous observations with conjugates labeled with 125I-HaLA. In the latter case, the degradation of high molecular mass conjugates by the 26 S protease and ATP was not accompanied by significant accumulation of lower derivatives (Fig. 7). It appears reasonable to assume that the lower molecular mass derivatives of 125I-Ub-labeled conjugates are protein-free polyUb chains that are formed by the action of the protease on the protein moiety of polyUb-protein conjugates. Incubation of conjugates with the 26 S complex in the absence of ATP also produced the same derivatives, but to a lower extent than in the presence of ATP. Treatment with isopeptidase in the second incubation caused the conversion of much of the 20-150 kDa derivatives (which accumulated in the first incubation with the 26 S complex + ATP) to free Ub. A reasonable interpretation appears to be that protein-free polyUb chains are preferred substrates for isopeptidase T (see “Discussion”).

It should be noted that a smaller amount of free Ub was formed in incubations with the isopeptidase in all cases, presumably due to incomplete action of the isopeptidase in the prior treatment. In addition, a small amount of free Ub was produced in the first incubation with the 26 S complex + ATP without the isopeptidase. This may be due to the presence of an isopeptidase in purified preparations of the 26 S complex or to the formation of a product that is indistinguishable from free Ub by size on SDS-polyacrylamide gel electrophoresis.

DISCUSSION

The isopeptidase studied in this work is a monomeric 100-kDa Ub-binding protein abundant in reticulocytes and erythrocytes. It acts preferentially on Ub-Lys4'-Ub linkages in polyUb chains (Fig. 3). Although the exact stoichiometry of Ub binding has not been determined in this study, it seems that the enzyme contains at least two Ub-binding sites, one of which has a high affinity for Ub aldehyde (Fig. 5). It is possible that the two sites bind two adjacent Ub units in a polyUb chain, between which is the isopeptide bond to be cleaved. An alternative, more unusual possibility is that the enzyme contains two active sites, only one of which is susceptible to inhibition by Ubal. That both sites coexist on the same enzyme is shown by the cooperativity between the binding of Ubal and the rate of exchange of bound Ub (Fig. 5).

The role of the enzyme in protein breakdown was revealed by the finding that the isopeptidase stimulates protein degradation in a reconstituted system (Fig. 6). The extent of the stimulatory effect of the isopeptidase was increased at high levels of E, and at limiting concentrations of the 26 S complex. High levels of E, promote the formation of excessively high molecular mass polyUb-protein conjugates (data not shown). It seemed possible that the degradation of such conjugates creates a difficulty for the 26 S complex and that this difficulty is overcome by the action of the isopeptidase. A relationship between the actions of the isopeptidase and 26 S complex was indeed shown by the finding that polyUb-protein conjugates are degraded in a synergistic fashion by the two enzymes in the presence of ATP (Fig. 7). Two types of interactions were found: (a) the isopeptidase stimulates the proteolysis of Ub-conjugated proteins by the 26 S complex (Fig. 8); and (b) the protease complex converts polyUb-protein conjugates to derivatives susceptible to the action of the isopeptidase (Tables II and III).

The above observations are consistent with the sequence of events shown in Fig. 10, in which the concerted action of the isopeptidase and 26 S protease leads to the breakdown of polyUb-protein conjugates. The isopeptidase acts on polyUb-protein conjugates with the liberation of free Ub and the formation of products designated polyUb* -protein conjugates (Fig. 10, Step 1). Two properties of the action of the isopeptidase ensure that the product remains to be a substrate for the 26 S protease complex. The enzyme does not cleave isopeptide linkages between polyUb chains and the protein substrate, as shown by the lack of liberation of free protein from polyUb-protein conjugates (Fig. 4). In addition, following the conversion of a portion of the high molecular mass conjugates to medium-sized conjugates, the remaining products are not susceptible to further action of this isopeptidase (Fig. 4). This may reflect a protective mechanism that prevents the complete disassembly of polyUb-protein conjugates prior to protease action. The nature of this protective mechanism is unknown. It may be that at certain sites of polyUb structures, isopeptide bonds exist at which linkage is to a Lys residue other than Lys4'. That polyUb chains may indeed contain other than Lys4' linkages is indicated by the observation that such chains are formed with UbR48 (Fig. 3). Since the isopeptidase acts preferentially on Ub-Lys4'-Ub linkages, it may be that such non-Lys4' linkages protect polyUb chains from premature disassembly. Other explanations, such as sterically protected Lys4' linkages, are equally possible.

Following the ATP-dependent action of the 26 S protease on polyUb-protein (or polyUb*- protein) conjugates (Fig. 10, Step 2), derivatives are formed that are highly susceptible to the action of the isopeptidase (Fig. 10, Step 3). This is indicated by the two-stage experiment described in Table III. We assume that the derivatives are protein-free polyUb chains based on the finding that they are labeled with 125I-Ub, but not with 125I-HaLA (Fig. 7 and Table III). Thus, an important role of isopeptidase T may be the removal of polyUb remnants that are the products of Ub-dependent proteolysis. The high levels of isopeptidase T in cells may be required for the recycling of the large amounts of polyUb remnants that are produced by Ub-dependent protein degradation.

The scheme proposed in Fig. 10 explains how the complete disassembly of polyUb chains is prevented until the breakdown of the protein is completed. However, several observations remained unexplained. It is not clear why protein-free polyUb chains are good substrates for the isopeptidase. It may be that the digestion of the protein moiety of polyUb*-protein conjugates by the 26 S protease removes a steric hindrance.

Since only fragments of 125I-HaLA that contain Tyr residues are labeled, it is possible that "protein-free polyUb chains" are linked to short fragments of the protein substrate devoid of Tyr residues.

**Fig. 10. Proposed sequence of events in concerted action of isopeptidase T and 26 S protease.** See the text for details.
that prevents the action of the isopeptidase. It is also possible that isopeptidase T acts preferentially next to a terminal Ub unit that has a free C terminus exposed by the action of the protease complex. This is compatible with the observation that binding of Ub to the enzyme requires a functional C terminus. Another problem is the mechanism by which the isopeptidase stimulates the rate of protein breakdown. The recycling of free Ub from polyUb remnants is presumably required for the continued action of the complete proteolytic system. However, the degradation of polyUb-conjugated proteins is not dependent upon the regeneration of free Ub; and yet, it is also stimulated by the isopeptidase (Fig. 8). This effect of the isopeptidase cannot be explained by the conversion of polyUb-protein conjugates to a degradable form by their trimming with the isopeptidase since pretreatment of polyUb-protein conjugates with the isopeptidase stimulated only slightly the rate of its subsequent degradation by the 26 S protease complex (Fig. 9). An alternative possibility is that protein-free polyUb chains are strongly bound to the 26 S protease complex, and thus inhibit the action of the complex on polyUb-protein conjugates. In such cases, the isopeptidase would stimulate the 26 S protease by the removal of an inhibitory product. However, the rate of conjugate proteolysis does not level off much earlier in the absence of the isopeptidase than in its presence, as would be the case when an inhibitor accumulates (Fig. 8A). It may be that protein-free polyUb chains dissociate slowly from the 26 S protease; and thus, the rate of proteolysis in the absence of the isopeptidase is limited by the rate of product dissociation. The isopeptidase may accelerate the rate of the clearance of polyUb chains from the 26 S protease if these compounds are channeled directly from the protease to the isopeptidase. Obviously, much further study is required to elucidate the integration of the action of isopeptidase T with that of the 26 S protease complex. Also remaining to be investigated is the identity and mode of action of “terminal” isopeptidase(s) that cleave the linkage between Ub and Lys residues derived from the protein substrate.

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