A 26 S Protease Subunit That Binds Ubiquitin Conjugates*

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Ubiquitin-mediated proteolysis provides an important mechanism for regulating a variety of cellular processes. Ubiquitin-conjugated proteins are degraded by a 26 S protease that contains more than 30 different subunits. Of these, a single 50-kDa polypeptide, subunit 5, specifically binds ubiquitin-lysozyme conjugates. Binding is inhibited by short polymeric chains of ubiquitin but not by ubiquitin monomers or by lysozyme. In addition, subunit 5 binds free ubiquitin chains with efficient association requiring at least four ubiquitins. Thus, proteins conjugated to polymers of ubiquitin may be selected for degradation by a single subunit of the 26 S protease complex.

Cell cycle events (1–3), photoperiodism in plants (4), and gene expression in yeast (5, 6) have all been shown to be regulated by ubiquitin-mediated proteolysis. Three classes of enzymes are required to activate and subsequently transfer ubiquitin to intracellular proteins (7). The conjugated proteins, especially those attached to polymeric chains of ubiquitin (8), are then degraded by a 26 S ATP-dependent protease (9, 10). The 26 S enzyme consists of the multicatalytic protease or proteasome (11, 12) associated with a regulatory complex (13) composed of 15 or more additional proteins. Two subunits of the regulatory complex, S4 and S7, belong to a novel ATPase family (14, 15) that may select non-ubiquitinated substrates for destruction by the 26 S protease (16). To date, the subunit(s) that recognizes ubiquitinated proteins has not been identified. Here we show that a 50-kDa subunit (S5) of the 26 S protease binds ubiquitin-lysozyme conjugates. Furthermore, recognition by S5 requires only polymeric ubiquitin.

EXPERIMENTAL PROCEDURES

Source of Proteins—Human red cell lysate was prepared as described (14). Regulatory complexes were partially purified from human red blood cells by chromatography on DEAE Fractogel and gel filtration on HW55 (17). The multicatalytic protease was purified from human red blood cells by described procedures (14). Ub-lysozyme conjugates were prepared by the method of Hough and Rechsteiner (18). Polyubiquitinated proteins were prepared by the method of Pickart et al. (19). Oxidized lysozyme used for competition was obtained by chloramine-T iodination in the presence of non-radioactive iodine.

Electrophoretic Procedures and Conjugate Binding Assays—Native gel electrophoresis was described in Hoffman et al. (13). High Tris SDS-PAGE was performed by the method of Fling and Gregerson (20). After native or SDS-PAGE, proteins were transferred to nitrocellulose membranes (21), incubated for 2 h in Tris-buffered saline (TBS) containing 5% nonfat dried milk, 0.05% Tween 20, and 0.02% NaN₃, and then washed for 10 min in TBS prior to a 1.5-h incubation with Ub-¹²⁵I-lysozyme conjugates at 4 °C. Membranes were briefly rinsed in TBS and allowed to dry prior to PhosphorImager analysis or exposure to x-ray film.

RESULTS AND DISCUSSION

Because numerous attempts to photocross-link Ub to the native 26 S protease were unsuccessful, we separated human red blood cell 26 S protease, regulatory complexes, and the multicatalytic protease on native gels, transferred them to nitrocellulose, and incubated the nitrocellulose strips with Ub-¹²⁵I-lysozyme conjugates. As shown in Fig. 1, A and B, we observed binding of the labeled conjugates to the intact 26 S protease and to regulatory complexes of the 26 S enzyme. The conjugates did not bind the multicatalytic protease (Fig. 1C) or several major proteins present in extracts of human red cells (Fig. 1, A and B). Strikingly, when regulatory complexes were subjected to SDS-PAGE and transferred to nitrocellulose, Ub-¹²⁵I-lysozyme conjugates bound a single 50-kDa subunit (Fig. 1D). Based on previous notation (14, 15), the conjugate-binding protein corresponds to subunit 5 (S5) of the 26 S protease. Densitometric analysis shows that S5 comprises more than 6% of the protein in the regulatory complex, an amount consistent with the presence of one subunit per complex. Close inspection of Fig. 1D reveals small amounts of Ub-¹²⁵I-lysozyme conjugates bound to proteins with apparent molecular masses of 36 and 35 kDa (asterisks in Fig. 1D). Since conjugate binding to these minor components increases upon storage of the 26 S protease, we suspect that the 36- and 35-kDa proteins are breakdown products of S5.

The Ub-¹²⁵I-lysozyme conjugates used in these experiments contain trace amounts of free ¹²⁵I-lysozyme (Fig. 2A, 14-kDa species in lane 1) raising the possibility that S5 binds ¹²⁵I-lysozyme rather than Ub-¹²⁵I-lysozyme conjugates. To identify the bound molecules, nitrocellulose slices containing S5 and bound radiolabel were heated to 80 °C in sample buffer, and the released proteins were analyzed by SDS-PAGE. Lane 2 of Fig. 2A shows that ¹²⁵I-lysozyme was not present among the radioactive molecules bound to S5; PhosphorImager analysis indicates slight enrichment for higher molecular weight conjugates (Fig. 2B).

The basis for Ub-lysozyme binding to S5 could be features inherent in monomeric ubiquitin, higher order structural features of multi-Ub chains, or features specific to the Ub-lysozyme isopeptide bond. Competition as well as direct binding experiments were used to address this question. Monomeric Ub at 267 μg/ml (31 μM) had little effect on the binding of Ub-lysozyme conjugates, suggesting that S5 does not efficiently recognize free ubiquitin; similar results were obtained with unconjugated lysozyme (Fig. 3A). In contrast, a Ub trimer linked via the C terminus and Lys-48 of successive ubiquitins competed strongly with Ub-lysozyme conjugates for binding to S5 (Fig. 3, A–C). At 120 μg/ml trimeric Ub caused a 50% reduction in Ub-lysozyme binding, consistent with an apparent

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1 The abbreviations used are: S5, subunit 5 of the 26 S protease; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; Ub, ubiquitin.
Ubiquitin Binding Subunit

**Fig. 1. Identification of a 26 S protease subunit that binds ubiquitin-lysozyme conjugates.** Human red cell lysate (A), partially purified regulatory complexes (B), or purified multicatalytic protease (C) were electrophoresed under non-denaturing conditions. Duplicate samples of each were assayed for peptidase activity (Act), stained with Coomassie Brilliant Blue (Prot), or transferred to nitrocellulose and incubated with anti-ubiquitin antibodies (21). The arrow denotes the 50-kDa polypeptide, subunit 5, that binds conjugates. 26 S identifies the 26 S protease. MCP is an abbreviation for Regulatory Complex, and R.C.* is a faster migrating form of the regulatory complex depleted of subunits S2 and S4 (see text). MCP stands for multicatalytic protease.

**Fig. 2. Molecular weight distribution of Ub-1251-I-lysozyme conjugates bound to S5.** Panel A, autoradiograms of labeled conjugates separated by SDS-PAGE prior to (lane 1) and after elution from S5 bands (lane 2). Panel B, PhosphorImager analysis of the molecular weight distribution of labeled conjugates before and after binding to S5. Regulatory complexes (5 µg/lane) were separated by SDS-PAGE on 10-20% high Tris gels, transferred to nitrocellulose, and incubated with labeled conjugates as described under “Experimental Procedures.” After rinsing, the S5 regions in three lanes were excised from the nitrocellulose, and bound radioactivity was removed by heating in SDS sample buffer at 80 °C for 10 min. The released molecules were then analyzed by SDS-PAGE on a 10-20% high Tris gel. Each S5 band contained approximately 130 cpm of Ub-1251-I-lysozyme conjugates. Approximately 200 cpm were electrophoresed in each lane shown above. Conjugates were visualized by exposure to x-ray film for 15 days.

$K_{0.5}$ of about 5 µM. Significant competition by the trimer is also apparent in Fig. 3B ($K_{0.5}$ ~9 µM). It should be noted that these apparent dissociation constants are upper limits, since the Ub trimer is competing with a heterogeneous pool of multi-ubiquitinated lysozyme conjugates, whose individual concentrations and affinities are unknown (see below). Dimeric Ub was also tested as a competitor; it was somewhat less effective than trimeric Ub. Results for 267 µg/ml of Ub monomer, dimer, and trimer (31, 16, and 10 µM, respectively) are presented in Fig. 3C.

Why dimeric Ub binds more weakly than trimeric Ub to S5 is currently unknown. However, differences in the three-dimensional structures of these species seem likely to contribute to this difference. The x-ray crystal structure of Ub dimer shows a compact molecule stabilized in part by sequestration of surface hydrophobic residues on each monomer in a pocket between the two ubiquitins (22). The tetramer is a more flexible and open structure consisting of a potentially infinitely repeating dimeric unit distinct from that of the isolated Ub dimer (23). Stabilizing Ub-Ub contacts in the tetramer are electrostatic in character. Although the structure of trimeric Ub has not been determined, it seems likely that this species has a tetramer-like structure since a third Ub added to the isolated dimer structure will have little opportunity for stabilizing interactions.

In view of these considerations, we explored the length dependence for the binding of Ub chains to S5. A mixture of radioiodinated Lys-48-linked chains ($n = 2–8$) was used to probe binding to SDS-PAGE separated S5. Binding of $^{125}$I-Ub polymers was readily detected by autoradiography (not shown). The bound species were then eluted with SDS-PAGE sample buffer and analyzed on 10-20% acrylamide gradient gels. Lane 2 of Fig. 3D indicates that S5 efficiently bound Ub polymers of $n = 4$ and higher. Since chains of $n > 4$ were rare relative to dimer and trimer (see Fig. 3D, lane 1), S5 must have a correspondingly higher affinity for longer chains. Such a specificity for S5 would provide a basis for the known preference of the 26 S protease for multi-ubiquitinated conjugates (8). At present there is no reason to think that longer chains have an intrinsically different structure from tetra-Ub. Thus S5 appears to bind Ub polymers in a manner highly cooperative with respect
increased local density of S5 subunits on the nitrocellulose membrane after transfer from denaturing gels may allow Ub conjugates to associate with more than one S5 molecule. It may appear surprising that S5 recognizes Ub-\(^{125}\)I-lysozyme conjugates after its transfer from SDS-PAGE gels, but calculations show that only 1 in 30 molecules of S5 needs to be renatured to account for the observed binding.

Additional 26 S subunits may recognize features of Ub conjugates other than Ub-Ub linkages. In this regard, Eytan et al. (24) recently presented evidence for an energy-dependent isopeptidase in the 26 S protease. Presumably, this de-ubiquitinating enzyme recognizes the Ub-substrate junction since bond cleavage occurs there. Subunit 5 of the 26 S protease could be an energy-dependent isopeptidase, but the direct binding of polymeric Ub species shown in Fig. 3D demonstrates that Ub-substrate isopeptide bonds are not required for conjugate recognition by S5.

Available peptide sequences from S5 indicate that the protein is not a member of the S4 ATPase family. Further characterization of Ub conjugate recognition by S5 will be facilitated by cloning and expressing a CDNA for the subunit. Subsequent molecular recognition studies should be straightforward since S5 can bind Ub conjugates even after its separation on SDS-PAGE gels.

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