Inhibition of the 26 S Proteasome by Polyubiquitin Chains Synthesized to Have Defined Lengths*

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Ubiquitin is a covalent signal that targets cellular proteins to the 26 S proteasome. Multiple ubiquitins can be ligated together through the formation of isopeptide bonds between Lys48 and Gly76 of successive ubiquitins. Such a polyubiquitin chain constitutes a highly effective proteolytic targeting signal, but its mode of interaction with the proteasome is not well understood. Experiments to address this issue have been limited by difficulties in preparing useful quantities of polyubiquitin chains of uniform length. We report a simple method for large scale synthesis of Lys48-linked polyubiquitin chains of defined length. In the first round of synthesis, two ubiquitin derivatives (K48C-ubiquitin and Asp77-ubiquitin) were used as substrates for the well characterized ubiquitin-conjugating enzyme E2-25K. Diubiquitin blocked at the nascent proximal and distal chain termini was obtained in quantitative yield. Appropriately deblocked chains were then combined to synthesize higher order chains (tetramer and octamer in the present study). Deblocking was achieved either enzymatically (proximal terminus) or by chemical alkylation (distal terminus). Chains synthesized by this method were used to obtain the first quantitative information concerning the influence of polyubiquitin chain length on binding to the 26 S proteasome; this was done through comparison of different length (unanchored) polyubiquitin chains as inhibitors of ubiquitin-conjugate degradation. Kcat was found to decrease ~90-fold, from 430 to 4.8 μM, as the chain was lengthened from two to eight ubiquitins. The implications of these results for the molecular basis of chain recognition are discussed.

The conserved protein ubiquitin functions in diverse biological processes, including oncogenesis, cell cycle progression, antigen presentation, and programmed cell death (for review, see Refs. 1 and 2). These and most other functions of ubiquitin reflect its role as an essential cofactor in an energy-dependent proteolytic pathway whose hallmarks are an unusual combination of high volume and high selectivity: most short lived proteins are degraded in this pathway, but the half-lives of individual substrate proteins can be regulated acutely and independently. Ubiquitin acts as a degradation signal by virtue of covalent ligation to target proteins. Ubiquitination occurs through the formation of an isopeptide bond between the COOH terminus of ubiquitin (Gly76) and an internal Lys residue of the target protein. This modification confers recognition by the multisubunit 26 S proteasome; the target protein is degraded, but ubiquitin is regenerated for use in subsequent proteolytic cycles (1–3). Specificity in ubiquitin-mediated proteolysis appears to arise primarily in the ubiquitin attachment step, which involves the sequential formation of ubiquitin thiol ester adducts of ubiquitin-activating (E1), conjugating (E2), and ligase (E3) enzymes (1, 4, 5). Recent studies suggest that for a given substrate, a specific E3 carries out the substrate ubiquitination step, whereas a specific E2 charges the E3 with ubiquitin (5).

The ligation of multiple ubiquitins increases the rate of substrate degradation (6–8), although the exact nature of the dependence is unclear. Multiple ubiquitination can occur through the ligation of ubiquitin monomers to several substrate Lys residues (9) but more typically involves the assembly on the substrate of a polymeric, isopeptide-linked ubiquitin chain (10). Multiple Lys residues of ubiquitin, including Lys6, Lys11, Lys29, Lys48, and Lys63, can serve as sites of polyubiquitin chain initiation and/or elongation (10–15). However, Lys48-linked chains represent the most commonly utilized degradation signal in the ubiquitin pathway. This conclusion is supported by several lines of evidence, including the results of in vitro biochemical analyses (10, 16) and the lethality of the K48R mutation in Saccharomyces cerevisiae (12). The targeting ability of Lys48-linked polyubiquitin chains apparently arises from their high affinity for the 26 S proteasome, which may be due in part to the exposure of a regular array of hydrophobic patches on the chain surface (17).

The 26 S proteasome is assembled from catalytic (20 S) and regulatory (19 S) subcomplexes (3, 18–20). The crystal structures of 20 S proteasomes from archaeaebacteria and yeast show that access to the proteolytic active sites is highly restricted (21, 22). Thus the target protein must be unfolded before degradation. Targeting of the ubiquitinated substrate to the proteasome is an activity of the 19 S complex, as suggested by the

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1 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; Uba1, ubiquitin aldehyde; IPTG, isopropyl-β-d-thiogalactopyranoside; H2S, hexahistidine; DTT, dithiothreitol; Bis-Tri, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; Ub, polyubiquitin chain composed of n ubiquitins; ATP-γS, adenosine 5′-O-(thiotriphosphate).

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ubiquitin independence of protein degradation catalyzed by the 20 S proteasome (3, 18–20). The 19 S complex contains a polyubiquitin chain-binding protein known as S5a, MBP1, or MCB1 (23, 24), and multiple subunits harboring ATP binding sites (3, 25). However, most of the subunits of the 19 S complex are functionally uncharacterized. One or more of these subunits must be an additional polyubiquitin-binding protein because a yeast mcb1Δ strain is viable and competent in ubiquitin-mediated proteolysis (26).

Biochemically useful quantities of substrates, i.e. polyubiquitinated target proteins, are a prerequisite for dissecting the mechanistic coordination of chain recognition, substrate unfolding, and peptide bond hydrolysis by the 26 S proteasome. Ideally these substrates should be homogeneous by several different criteria: overall purity, polyubiquitin chain length, chain linkage, and site of chain ligation to the target protein. So far, such well defined substrates have been unattainable because of the low purity and abundance of E3 enzymes, as well as the uncontrolled character of chain elongation as catalyzed by available E2 and E3 enzymes. To overcome the chain elongation problem, we have implemented a novel method that utilizes the well characterized ubiquitin-conjugating enzyme E2-25K to generate Lys48-linked polyubiquitin chains of defined length. This method avoids uncontrolled polymerization by utilizing, in each round of synthesis, two chain reactants, one reversibly capped at its proximal, and the other at its distal, terminus. This method generates “unanchored” chains that bind to the 26 S proteasome and inhibit the degradation of polyubiquitinated lysozyme. This inhibition assay was used to evaluate, for the first time, the relative binding of different length chains to the 26 S proteasome.

**EXPERIMENTAL PROCEDURES**

**Ethyleneimine was purchased from ChemService, stored at 5 °C, and used within several days of opening the sealed ampule. Except where noted, other reagents were purchased from Sigma. Protein iodoination with chloramine T was carried out as described (4). Trypsin digestion of ubiquitin derivatives (5 mg/ml total ubiquitin and 5% w/w trypsin) and reversed phase high performance liquid chromatography separation of peptides were carried out as described (27, 28).**

**Ubiquitin Hydrolyase-1 was prepared by a slight modification of the method described previously (31). Ubal was prepared by either of two methods described previously (32, 33).**

**Analysis of Alkylation Kinetics—**Kinetics were monitored at 37 °C in incubations containing 0.2 mM Tris-HCl (50% base), 1 mM EDTA, 1 mM cysteine, or 0.5 mM K48C-ubiquitin, and 0–64 mM alkylating agent; or (Ph trial) (8.5). At timed intervals, aliquots were assayed for remaining thiol with dithionitrobenzole (37). Values of kobs were obtained from linear plots of ln A/Ao against time and were corrected by subtracting the value of kobs measured in the absence of alkylating agent.

**Preparative Alkylation (Distal Terminus Deblocking)—**For the experiments shown in Table I and Fig. 3, conditions were as follows. Reactions with ethyleneimine contained 50 mM ethyleneimine and the purified ubiquitin chain at 2–20 mg/ml (≤1 ml). Other conditions were the same as for kinetic measurements (above). After 1 h, the reaction mixture was dialyzed against 5 mM ammonium acetate (pH 5.5), 0.1 mM EDTA, 1 mM DTT to remove residual ethyleneimine. Addition of K48C-ubiquitin (1 mg/ml) with bromoethylamine (50 mM, Aldrich) was carried out similarly. For alkylation of K48C-ubiquitin (1 mg/ml) with N-(N-dodecyl)-trifluoracetamide (Aminoethyl-87°, Pierce), 3.4-h incubations were done at 50 °C in 0.2 mM Bis-Tris propane (as above). The alkylation agent was dissolved in methanol and added in two equal portions at time zero and 1 h to give a final concentration of 3.1 mM. After each addition of DTT to 2.0 mM, the reaction was dialyzed as above.

**Proximal Terminus Deblocking—**The purified ubiquitin chain (2–50 mg/ml, ≤1 ml) was incubated with 15 µg yeast ubiquitin hydrolyase-1 for 1 h at 37 °C in 50 mM Tris-HCl (24% base), 0.1 mM EDTA, and 0.5 mM DTT. The reaction was passed through a 0.5-ml Q-Sepharose column (Pharmacia; preequilibrated in the same buffer) to absorb the enzyme; the flow-through was collected into a Centricon-10 microconcentrator (Amicon). The column was washed with 3 volumes of buffer.

The combined flow-through and wash fractions were concentrated.

**Protein Degradation Assays—**The production of acid-soluble radioactivity from 125I-lysozyme or 125I-lactalbumin (~106 cpm/µg; ~105 cpm/25-µl incubation) was monitored in incubations with rabbit reticulocyte fraction II (~1.5 mg/ml protein) as described (17). Rates obtained in the presence of wild type ubiquitin or ubiquitin derivative (12 µM) were corrected using blanks obtained by omitting ubiquitin. In some cases aliquots were withdrawn during the steady state of degradation for analysis of the levels of ubiquitin-substrate conjugates after electrophoresis and autoradiography (17). For the experiment involving disassembly of engineered Ub (see the last entry in Table I), 4.5 µM Ub3 (potentially yielding 15 µM monoubiquitin) was preincubated for 30 min under the conditions of the degradation assay, including the denaturating system, and labeled substrate were omitted. These components were added to initiate the assay. For the control, the preincubation contained 18 µM wild type monoubiquitin.

**Engineered Polyubiquitin Chains: Synthesis and Purification —**Inclusions of 0.2–2.0 µl contained 50 mM Tris-HCl (50% base), 5 mM MgCl2, 10 mM phosphocreatine, 0.6 unit/ml each of creatine phosphoki...
nase and inorganic pyrophosphatase, 4 mM ATP, and 0.5–1 mM DTT (37 °C); E1 and C1705,F174L-E2-25K were 0.2 mM and 20 μM, respectively. The two chain reactants were added at equal concentrations. Times required for completion of the conjugation reaction, usually 1–4 h, were determined by examining reaction aliquots by SDS-polyacrylamide gel electrophoresis. At the end of the incubation, the reaction was passed through a Q-Sepharose column (0.5–1 ml) at pH 7.6 to absorb the enzymes. In most cases the unabsorbed fraction was adjusted to pH 4.5 (for non-tagged chains) or 6.1 (for H6-tagged chains) and applied to an S-Sepharose column pre-equilibrated at the appropriate pH (Pharmacia; 15 mg of ubiquitin/ml of resin), and the major chain product was purified by elution with a linear gradient of NaCl (0–0.5 M). Fractions spanning the peak region were examined by SDS-polyacrylamide gel electrophoresis and Coomassie staining before pooling. Concentration and buffer exchange were carried out using Ultrafree-4 or -15 devices (Millipore). Ub4 and Ub3 were resolved by gel filtration on a 1 × 50-cm column of Sephadex G-75 (Sigma) buffered with 50 mM ammonium acetate (pH 5.5), 0.1 mM EDTA, and 1 mM DTT.

Wild Type Polyubiquitin Chains—For Ub4, the incubation (0.25 ml) was carried out as described for engineered chains (above), except that the reactants were K48R-ubiquitin (3 mg/ml) and Ub3 assembled from wild type ubiquitin (~12 mg/ml). The Ub4 was largely des-Gly-Gly at its proximal terminus. After 2 h of incubation (37 °C), E1 and E2-25K were removed by passage through an anion exchange column (above), and Ub4 was purified by gradient elution (above) from a fast protein liquid chromatography Mono S column (Pharmacia). Cation exchange was also used to resolve Ub4 and Ub3 from mixed chains assembled from wild type ubiquitin (38).

Preparation of H6-Ubiquitin-125I-Lysozyme Conjugates—Incubations of 2 ml contained (pH 8.0, 37 °C) 50 mM Tris-HCl (24% base), 5 mM MgCl2, 5 mM ATP, 0.05% boehringer Mannheim, 0.8 μM Ub4, 10 μM DTT, 0.3 mM inorganic pyrophosphatase, 3.3 mg/ml fraction II protein, 0.3 mg/ml H6-ubiquitin, and ~4 × 107 cpm of 125I-labeled-lysozyme (40–50 μg). After 2 h, 20 μM N-ethylmaleimide (to quench DTT and inactivate the enzymes) was added together with 4 mM urea and 10 mM imidazole. The quenched incubation was applied to a 1-ml column of Ni-NTA agarose (Novagen) preequilibrated with buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10% v/v glycerol, 10 mM DTT, 1.5 or 3 mM Ubal, 2 mM ATP, 10 mM phosphocreatine, 0.3 unit/ml creatine phosphokinase, 0.3 unit/ml pyrophosphatase, 0.37 mg/ml 26 S proteasome (0.18 μM based on a molecular mass of 2.1 MDa), and ~5,000–8,000 cpm of 125I-lysozyme conjugates (~30 μg lysozyme; above). The lysozyme conjugates also contributed H6-ubiquitin at a final concentration of ~10 μM. Only a small fraction of this H6-ubiquitin was conjugated to 125I-labeled-lysozyme of the remainder, some was unconjugated, and some was conjugated to fraction II proteins (the conjugated/unconjugated ratio is unknown). Ubal was included to inhibit disassembly of polyubiquitin chains by one or more isopeptidases in the 26 S proteasome preparation; control experiments showed that 1.5–3 μM Ubal completely inhibited polyubiquitin chain disassembly but had no effect on the rate of conjugate degradation. Incubations were quenched after 10 min by the addition of trichloroacetic acid, and acid-soluble radioactivity was determined by counting an aliquot of the acid supernatant for 10 min. Appropriate controls showed that degradation was linear with time for ~15 min and that degradation was abolished if MgATP was omitted.

Because initial experiments showed that the addition of ubiquitin chains to the assay significantly increased the recovery of acid-soluble radioactivity by counteracting nonspecific absorption, all assays included monoubiquitin as carrier. Most assays were supplemented with 230 μM monoubiquitin; in some cases, the concentration was 420 μM. Data from the two sets of experiments could be fit by the same K50 value for Ub5, suggesting that monoubiquitin did not bind competitively with unanchored polyubiquitin chains. This was confirmed by showing that ~50% inhibition by 29 μM Ub5 (see “Results”) was observed whether or not the assay was supplemented with 230 μM monoubiquitin.

Mass Spectrometry—Matrix-assisted laser desorption time-of-flight mass spectrometry employed a PerSeptive Biosystems Voyager RP spectrometer operated in linear mode at an accelerating voltage of 30 kV. Samples were prepared with either α-cyano-4-hydroxycinnamic acid or 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix compound, and the instrument was calibrated by use of the single-charged (m/z = 14,314.2) and double-charged (m/z = 7,157.6) cations from an internal standard of chicken egg white lysozyme.

RESULTS AND DISCUSSION

Principle of the Method—Fig. 1 shows the method for synthesizing Lys48-linked polyubiquitin chains of defined length. The approach utilizes ubiquitin-conjugating enzyme E2-25K to generate a Lys48-Gly76 isopeptide bond between two ubiquitin

![Diagram](image_url)
derivatives. One of these derivatives is capped at its COOH terminus (future proximal chain terminus) by the presence of an extra residue, Asp\(^{77}\). The other ubiquitin derivative is capped at the ubiquitin-accepting site (future distal chain terminus) by the presence of a Lys-to-Cys mutation at residue 48. In the first round of synthesis, these reactants are the two monomeric derivatives Asp\(^{77}\)-ubiquitin and K48C-ubiquitin. In principle, E2-25K should quantitatively convert these two reactants to Ub\(^{3}\); once Gly\(^{76}\) of K48C-ubiquitin is linked to Lys\(^{48}\) of Asp\(^{77}\)-ubiquitin, further polymerization is prevented by the absence of additional Lys\(^{48}\) and Gly\(^{76}\) residues. Additional rounds of controlled conjugation are possible because the proximal or distal terminus of the double-capped dimer is easily deblocked; Asp\(^{77}\) can be removed catalytically by the ubiquitin-processing protease yeast ubiquitin hydrolase-1 (31), whereas chemical alkylation of Cys\(^{48}\) with ethyleneimine (below) creates a lysine mimic (39). The two single-deblocked dimers should be quantitatively converted to Ub\(^{3}\) in the next round of synthesis. By using the appropriate combination of single-capped chain reactants, this method can in principle give rise to a chain of any length.

**Efficiency and Specificity of Deblocking**—E2-25K catalyzes the synthesis of polyubiquitin chains harboring exclusively Lys\(^{48}\)-Gly\(^{76}\) isopeptide bonds (28, 38) and is available in recombinant form (40). E1 and E2 enzymes can transfer polyubiquitin chains (28, 41). Thus, there is no block to efficient and specific conjugation provided the necessary chain reactants are available. This is trivial in the first cycle of synthesis, which directly utilizes recombinant monomeric ubiquitins. In subsequent cycles, however, the efficiency of conjugation is highly dependent on the efficiency with which the double-capped product of the previous cycle is deblocked. In addition, the functionality of long chains generated by this method is likely to depend strongly on specificity in chemical alkylation; a low level of reaction with side chains other than Cys\(^{48}\) could be deleterious after the multiple rounds of alkylation needed to produce long chains.

We expected that yeast ubiquitin hydrolase-1-catalyzed proximal deblowing would proceed with high efficiency and specificity, and this proved to be the case. Removal of the proximal Asp\(^{77}\) residue was very rapid, proceeding to completion within 60 min in reactions involving mono-, di-, and tetrameromic ubiquitin derivatives, at concentrations as high as 50 mg/ml. There was no evidence for cleavage of Lys\(^{48}\)-Gly\(^{76}\) isopeptide bonds. The single-deblocked Ub\(_{2}\) derivative resulting from yeast ubiquitin hydrolase-1 treatment (see below) was found to have a molecular mass of 17,090.5 ± 8.2 Da, which compared favorably with the calculated mass of 17,087.7 Da.

It seemed likely that efficiency and specificity would be more problematic in the alkylation of Cys\(^{48}\) than in the removal of Asp\(^{77}\). Thus we began with a systematic comparison of three reagents that could add an aminoethyl moiety to Cys\(^{48}\): ethyleneimine, bromoethylamine, and N-(iodoethyl)trifluoroacetamide (Aminoethyl-8TM). N-(Iodoethyl)trifluoroacetamide was used to alkylate K48C-ubiquitin in an earlier study (16), but the efficiency and specificity of alkylation were not determined. In initial studies we determined the rates at which moderate concentrations of the three reagents alkylated free cysteine.

The pseudo-first-order reaction observed with 64 mM ethyleneimine at pH 8.1 and 37 °C is shown in Fig. 2 (open circles), indicating that ethyleneimine did best alkylate the distal Cys\(^{48}\) in a chain reactant at a rate competitive with undesired alternative reactions, such as Cys\(^{48}\) oxidation. As shown in Fig. 2 (open circles), ethyleneimine reacted with Cys\(^{48}\) in K48C-monoubiquitin about two times more slowly than with free cysteine, presumably reflecting steric hindrance from surrounding side chains.

The efficiency and specificity of alkylation were examined further by tryptic peptide mapping of K48C-ubiquitin after reaction with each of the reagents discussed above (for conditions of the alkylation reactions, see “Experimental Procedures”). Trace a in Fig. 3 shows the map of wild type ubiquitin; peptides 4–11 are clearly resolved (27). The K48C mutation abolishes a tryptic cleavage site (top, Fig. 3); this is reflected as loss of peptides 4 and 8, concomitant with the appearance of a new combined peptide, labeled 12 in Fig. 3, which contains Cys\(^{48}\) (compare traces a and b). (The low yield of peptide 6 in trace b is not significant; peptides 5 and 6 are overlapping, and their relative yields were variable.) Because alkylation restores a cleavage site at residue 48, the extent of alkylation could be quantitated by the reappearance of peptides 4 and 8 and the disappearance of peptide 12. The peptide map of ethyleneimine-treated K48C-ubiquitin (trace c) shows essentially full recovery of peptides 4 and 8 and a corresponding loss of peptide 12 (Fig. 3, traces d and e).

Inspection of trace e further suggests that ethyleneimine did not react significantly with other potential nucleophilic side chains, such as Met\(^{1}\) and His\(^{68}\); modification of these side chains would probably be manifested as shifts in peaks 9 and 11, respectively (27). Mass spectrometric analysis of alkylated diubiquitin (bearing a proximal Asp\(^{77}\) residue, see below) confirmed this inference; the observed mass of 17,232.7 ± 12.4 Da compared favorably with the calculated mass of 17,245.8 Da. Taken together, these results indicate that ethyleneimine acts with high efficiency and specificity at the side chain of Cys\(^{48}\), in both mono- and dimeric ubiquitin derivatives.

**Functionality of Alkylated Ubiquitin**—To address the functionality of alkylated K48C-ubiquitin, we tested its ability to support degradation in a modified reticulocyte lysate, fraction
II (4), in which degradation is dependent upon the addition of functional ubiquitin. Ubiquitin derivatives that cannot form chains are expected to show impaired activity in this assay (10, 16). For the two substrates assayed, there was indeed a decreased rate of degradation when K48C-ubiquitin (or K48R-ubiquitin) was substituted for wild type ubiquitin (Table I), and this decreased rate correlated with a strongly reduced level of very high molecular weight conjugates (Fig. 4, lanes 3 and 4 versus 2). These conjugates presumably bear long polyubiquitin chains.

Alkylation of K48C-ubiquitin with ethyleneimine partially (125I-lactalbumin) or fully (125I-lysozyme) restored activity in degradation (Table I). As expected based on this result, alkylation with ethyleneimine also restored the formation of very high molecular weight conjugates of 125I-lactalbumin (as monitored by electrophoresis and autoradiography; Fig. 4, lane 5 versus 2). We do not know why full activity in 125I-lactalbumin degradation was not restored after alkylation. However, results with the other alkylating reagents (below) indicate that this is an intrinsic effect of the presence of the S-aminoethylcysteine moiety rather than a specific effect of ethyleneimine. Preliminary concentration dependence studies, carried out with ethyleneimine-treated K48C-ubiquitin, indicated that this was a V_max effect (data not shown). These results show that S-aminoethylcysteine at position 48 functions similarly to Lys in ubiquitination and conjugate degradation, confirming the conclu-

![Graph and Table]

**Table I**

Support of degradation by mutant ubiquitins and alkylated derivatives

| Ubiquitin                | 125I-Lactalbumin | 125I-Lysozyme |
|-------------------------|------------------|--------------|
| K48C-ubiquitin          | 37.8 ± 2.0       | 59.4 ± 3.7   |
| K48R-ubiquitin          | 9.3 ± 1.7        | 35.7 ± 8.2   |
| K48C (ethyleneimine)    | 65.6 ± 3.8       | 95.5 ± 5.2   |
| K48C (bromoethylamine)  | 70.5 ± 4.3       | 97.4 ± 5.3   |
| K48C (Aminoethyl-8)     | 62.9 ± 2.7       | 91.6 ± 5.1   |
| Disassembled Ub_4       | 68.8             | Not done     |
Inhibition of 26 S Proteasome by Polyubiquitin Chains

forms are also seen above the contaminant band. K48R-ubiquitin, two ubiquitinated forms of lactalbumin are visible below the contaminant band. Lys48 inhibited more strongly with 125I-lactalbumin than with bromoethylamine. Alkylated Ub4 was almost completely disassembled by endogenous isopeptidases during a 30-min incubation contained 20 mg of each reactant in 2 ml (pH 7.3). Lane 1, zero time; lane 2, 4 h. Lanes 3 and 4, Ub synthesis. The incubation contained 7 mg of each reactant in 0.7 ml (pH 8.0). Lane 3, zero time; lane 4, 1 h. Lanes 5 and 6, Ub2 synthesis controls. Double-capped dimer (10 mg/ml) was incubated for 1 h with 10 mg/ml dimer deblocked at the proximal terminus (lane 5) or at the distal terminus (lane 6) (pH 8.0). Lanes 7 and 8, Ub3 synthesis. The incubation contained 2 mg of each reactant in 0.6 ml (pH 8.0). Lane 7, zero time; lane 8, 1.5 h.

In a final experiment, we used alkylated Ub3 (resulting from two cycles of synthesis, Fig. 1) as the source of ubiquitin to support degradation. Alkylated Ub3 was almost completely disassembled by endogenous isopeptidases during a 30-min preincubation in fraction II (without ATP), as indicated by the results of Western blotting with anti-ubiquitin antibodies (not shown). After the addition of ATP, the monomers produced by disassembly supported degradation at the same rate as alkylated K48C-ubiquitin (Table I). This result provides a qualitative indication that isopeptidases recognize the modified isopeptide bond containing the S-alpha-ethylcysteine moiety and shows that two rounds of alkylidylation did not result in gratuitous inhibitory modifications. Based on the functionality of products generated using ethyleneimine (Table I and Fig. 4) and on the high efficiency and specificity of this reagent (Figs. 2 and 3), we selected ethyleneimine as the alkylating agent in large scale synthesis of polyubiquitin chains (Fig. 1 and below).

Two additional features of the functional data (Table I), although not directly relevant to our original objectives, deserve comment. First, the quantitative effect of blocking polyubiquitin chain formation was substrate-dependent; mutation of lys3-48 inhibited more strongly with 125I-lactalbumin than with 125I-lysozyme. Similarly, Hershko and co-workers (9, 43) reported that reductive methylation of ubiquitin, which blocks chain formation, suppresses 125I-lactalbumin degradation more strongly than 125I-lysozyme degradation. The stronger inhibition seen with 125I-lactalbumin probably reflects a difference in the rate-limiting step; conjugate formation is the slow step in 125I-lysozyme degradation (44), whereas conjugate degradation may be partly rate-limiting in 125I-lactalbumin turnover.6 Lactalbumin degradation is thus expected to be more sensitive to features such as chain length, which influence conjugate recognition by the 26 S proteasome. In addition, we cannot exclude the possibility that non-polyubiquitinated lysozyme conjugates are recognized better than comparable forms of lactalbumin.

Second, K48R- and K48C-ubiquitin were not equivalent: the K48C mutation was significantly less inhibitory with both substrates. In the case of 125I-lactalbumin, this difference was probably caused by the greater ability of K48C-ubiquitin to support the formation of conjugates bearing multiple single ubiquitins (up to n = 5; Fig. 4, lanes 3 versus 4). This effect may be explained by decreased susceptibility to de-ubiquitination because conjugates bearing K48C-ubiquitin, but not K48R-ubiquitin or alkylated K48C-ubiquitin, are resistant to disassembly by an isopeptidase associated with the 26 S proteasome (45).7 The inhibitory effect of a given chain-terminating mutation can also be influenced by substrate structure; with an engineered β-galactosidase substrate, both K48R- and K48C-ubiquitin inhibited degradation by more than 90% (10, 16). Here the similar behavior of the two mutant ubiquitins, and the very dramatic inhibition, may be explained by the presence of only a single ubiquitination site in the target protein (10); regardless of the nature of the mutation at residue 48, only monoubiquitinated β-galactosidase could be formed.

Large Scale Synthesis of Defined Length Polyubiquitin Chains—Synthesis of double-capped Ub2 from Asp77-ubiquitin and K48C-ubiquitin is shown in lanes 1 and 2 of Fig. 5. This 2-ml incubation, carried out at pH 7.3, contained 40 mg of total ubiquitin; dimer synthesis was complete in 4 h. The reaction was faster at pH 8, and this condition was used in subsequent synthetic reactions. Up to 60 mg of each monomer has been combined in such first-cycle reactions.

3 R. Beal, D. Toscano-Cantaffa, P. Young, and C. Pickart, manuscript in preparation.
4 Y. Lam, G. De Martino, C. Pickart, and R. Cohen, manuscript in preparation.
Inhibition of 26 S Proteasome by Polyubiquitin Chains

Synthesis of double-capped Ub₈ is shown in lanes 3 and 4 of Fig. 5. This 0.7-ml incubation, carried out at pH 8, contained 7 mg of each single-deblocked Ub derivative; synthesis was essentially complete in 1 h. Up to 40 mg of each dimeric derivative has been combined in second-cycle reactions. An additional round of deblocking and synthesis gave rise to double-blocked Ub₈ (Fig. 5, lanes 7 and 8).

In any given incubation, formation of the chain product was absolutely dependent upon the presence of both appropriately deblocked reactants, as shown for Ub₂ synthesis in lanes 5 and 6 of Fig. 5. This last result confirms the very high linkage specificity of E2-25K (29, 38). A trace of apparent Ub₈ in lane 4 (Fig. 5) may be because of the loss of Asp⁷⁷ from the proximally capped dimer catalyzed by an E. coli carboxypeptidase that is present in trace amounts in some E2-25K preparations. Synthesis was very efficient; when the concentrations of the input reactants were equal, there was nearly complete conversion to the expected product (Fig. 5, lanes 3, 4, and 7, 8).

Ub₄ and Ub₂ products were resolved from remaining reactants by gradient cation exchange chromatography; Ub₄ was purified by gel filtration (see “Experimental Procedures”). Typically, the major chain product was obtained in about 60% yield from cation exchange columns, although yields were sometimes as high as 80%. Recoveries from the deblocking reactions were higher: nearly 100% for the yeast ubiquitin hydrolase-1 reaction and ~90% for the ethyleneimine reaction.

Binding of Engineered Chains Inhibits Conjugate Degradation by the 26 S Proteasome—Because the rates of ubiquitination and de-ubiquitination, as well as the rate of conjugate degradation, may contribute to the overall rate of degradation in fraction II, it is difficult to draw quantitative conclusions about conjugate recognition based solely on steady-state measurements in this system (above). We showed previously that a mixture of unanchored Lys⁴⁸-linked chains inhibited overall degradation in fraction II, indicating that chains compete with polyubiquitinated substrates for binding to chain recognition components of the 26 S proteasome (17). A higher resolution version of this assay provided a convenient monitor of the binding of engineered chains to the proteasome. These experiments utilized purified mammalian 26 S proteosomes (18) and purified ¹²⁵I-lysozyme conjugates made with H₆-ubiquitin (see “Experimental Procedures”).

Ub₂ inhibited the purified proteasome with a hyperbolic concentration dependence that corresponded to K₀.₅ = 27.6 ± 4.3 µM (open circles, Fig. 6). Western analysis with anti-ubiquitin antibodies indicated that Ub₂ was stable during the assay (data not shown). Inhibition must thus be caused by competition with substrate conjugates. The presence of the proximal (Asp⁷⁷) and distal (Cys⁴⁸) blocking residues did not influence binding because similar inhibition was seen with double-blocked Ub₂ versus each of the two single-blocked species (at 29 µM; these data are included in the open circles in Fig. 6). Failure of the chain termini to influence binding is consistent with the expectation that substrate conjugates, which bear a macromolecule at the proximal chain terminus, will bind primarily through interaction with the polyubiquitin chain. A series of hydrophobic patches which has been implicated in the binding of chains to the proteasome lies on the surface of the chain (17); this is also consistent with the observed absence of end effects.

Fig. 6 (filled circle) also shows the effect of wild type Ub₄ at 29 µM. This chain had normal isopeptide linkages, with K₄₈R-ubiquitin at the distal position (to facilitate synthesis; see “Experimental Procedures”). If anything, the wild type tetramer inhibited more weakly than the engineered tetramers, confirming that the presence of one or two S-aminoethylcysteine moieties in the engineered tetramer was fully permissive for binding. This conclusion is consistent with the the results shown in Table I.

Length Dependence of Inhibition—The rate of conjugate degradation is known to be facilitated by the presence of multiple ubiquitins (6–8, 10, 16; Table I). In the case of conjugates bearing chains, degradation is thought to increase with chain length. This may be caused both by enhanced binding of long chains and their resistance to disassembly by a proteasome-associated isopeptidase (45). With regard to binding, no quantitative studies have addressed the form or basis of the presumptive length dependence. We show in Fig. 6 (inverted triangles) that Ub₄ inhibited the proteasome with a hyperbolic concentration dependence that corresponded to K₀.₅ = 4.8 ± 1.0 µM. This value is 5.8-fold smaller than the K₀.₅ for Ub₂. Given the errors in the respective measurements, the ratio could be as high as 8 but no lower than 4.

Inhibition by shorter chains was examined in a preliminary way. These chains were assembled from wild type ubiquitin; the absence of the S-aminoethylcysteine moiety should not influence binding (above). As shown in Fig. 6, 78 µM Ub₂ caused ~50% inhibition (square), whereas 117 µM Ub₄ caused only ~21% inhibition (diamond). The observed inhibition is consistent with K₀.₅ values of ~80 µM for Ub₄ and ~430 µM for Ub₂, assuming a hyperbolic concentration dependence as is seen with longer chains.

The affinity of polyubiquitin chains for the proteasome thus increases ~90-fold as n increases from 2 to 8 (Table I). This is substantially steeper than the chain length dependence observed in the proteasome-catalyzed degradation of a series of α-globin conjugates (45). In the latter study, there was a rate
increase of ~2-fold as n increased from 1 to 6 (degradation in the presence of Ubal (45)). This weaker dependence on n is probably caused by the structures of the α-globin conjugates, which are linked to monoubiquitin(s) at low n values, and by a mixture of monoubiquitins and short chains at higher n values (46). It is likely that conjugates linked to monoubiquitin interact with the proteasome differently than conjugates linked to polyubiquitin chains.

Our results bear on the mechanisms by which polyubiquitin chains may target substrates to the proteasome. The data unambiguously exclude a model in which the chain simply amplifies the local concentration of monoubiquitin because this model predicts that \( K_{0.5} \) will decrease in direct proportion to chain length; instead, \( K_{0.5} \) decreases by ~90-fold as n increases by 4-fold (Table II). A model in which monoubiquitin is the recognition element is also inconsistent with the failure of monoubiquitin to compete with polyubiquitin chains (see "Experimental Procedures"). The results are generally consistent with a model in which assembly of ubiquitin into a chain creates and amplifies a specific structural element that is recognized by one or more binding components in the 19 S regulatory complex. A satisfying feature of this model is that it could allow monoubiquitin to serve distinct functions from polyubiquitin chains (e.g. 47).

When immobilized on a nitrocellulose membrane, subunit S5a of the 19 S complex exhibits negligible binding of chains shorter than Ub4, whereas for chains of n ≥ 4 binding increases steeply with chain length (23). This behavior led to the suggestion that Ub4 harbors a minimum recognition element for S5a (17, 23). We considered whether our data could be consistent with a similar model for the proteasome. Scheme I shows a model of Ub8 which is based on the crystal structure of Ub4 (48). The scheme depicts a series of surface hydrophobic patches composed of the side chains of residues Leu8, Ile44, and Val70. Mutation of pairs of these residues to Ala abolishes binding of polyubiquitin chains to the proteasome and to isolated S5a, suggesting that these patches are part of a chain-based recognition element (17).

For the simple case in which Ub4 represents the actual recognition element, and longer chains are assumed to have the conformation seen in the Ub4 crystal, there are three elements in Ub6 (ubiquitins 1–4, 3–6, and 5–8 in Scheme I). This model predicts that Ub6 will bind three times more tightly than Ub4; this factor is close to the lower limit of 4 which can be accommodated by our binding data (above). The model is also consistent with the observed negligible inhibition by Ub4. Significant inhibition by Ub4, although not predicted, might arise if the conformation of Ub4 is Ub4-like, and a partial recognition element is partially functional. A related model, in which ubiquitins 2–5 and 4–7 (Scheme I) are considered to be identical to the elements specified above, predicts that Ub4 will bind five times more tightly than Ub4; this prediction is in good agreement with the data.

### Table II

| N       | Observed value | Relative value |
|---------|----------------|----------------|
| 2       | 430            | 89.6           |
| 3       | 80             | 16.7           |
| 4       | 27.6 ± 4.3     | 5.8            |
| 8       | 4.8 ± 1.0      | 1              |

### Scheme I. Model of Ub8 which is based on the crystal structure of Ub4 (48). The scheme depicts, as small circles, a series of surface hydrophobic patches composed of the side chains of residues Leu8, Ile44, and Val70. The solid circles are patches facing the viewer; the stippled circles are patches on the opposite face of the chain.

An alternative model postulates that two adjacent hydrophobic patches on the same face of the chain represent a minimal recognition element. The two ubiquitins bearing these patches, e.g. ubiquitins 2 and 4 in Scheme I, are not directly covalently linked. Therefore Ub2 lacks a recognition element and should cause negligible inhibition, as observed. Ub3, if its conformation is Ub4-like, contains one element, Ub4 contains two, and Ub8 contains six. Ub3 inhibits about two times more weakly than predicted by this model; this could reflect the inability of Ub3 to replicate faithfully the conformation seen in the Ub4 crystal (48). Like the previous model, this model predicts that the affinity of Ub8 should be three times that of Ub4, which is close to the lower limit of 4 which can be accommodated by the current results (above).

These considerations serve to indicate that the enhanced interaction of longer chains with the proteasome can be explained by a model in which the structure of the chain serves to create and amplify a minimum binding element that includes two or more hydrophobic patches. Studies on the interaction of longer chains (n > 8) and mutant chains (below) with the proteasome will be helpful in defining the precise nature of this element or in developing alternative models. As noted above, the observed ratio of \( K_{0.5} \) values for Ub4 and Ub8 is somewhat larger than the ratio predicted by two of the models discussed above. However, "fraying" of the ends of the chain, which probably occurs in solution, would disproportionately weaken the binding of short chains, leading to an increase in this ratio.

In previous work we synthesized several Ub4 molecules bearing L8A, I44A-ubiquitin at one or more defined positions within the chain. Because chains assembled solely from this mutant ubiquitin cannot target substrates for degradation (17), such defined oligomers may provide a way to test the models outlined above. We measured binding of these molecules to S5a immobilized on a nitrocellulose membrane; the results (17) agreed best with a four-ubiquitin recognition element. However, the poor sensitivity of this binding assay, and the finding that the yeast S5a homolog is nonessential (26), indicate that these issues should be re-addressed with the 26 S proteasome. Application of the method described here will facilitate the assembly of mixed wild type/mutant polyubiquitin chains.

Ubiquitin is present in cells at a concentration of ~20 μM, of which about half is in the form of conjugates (49). Although the length of the chain on a typical conjugate undergoing degradation is not known, substrate-linked chains of n > 8 are difficult to detect (e.g. 10, 16). Thus, it is reasonable to expect that Ub8 should constitute an efficient targeting signal. The question arises as to whether the binding reported here for Ub8 (\( K_{0.5} \) ~ 4 μM, Fig. 6) is sufficient to allow rapid degradation of polyubiquitinated proteins in vivo. This question cannot be answered with certainty, but it is probable that affinities only modestly higher than those observed would be adequate be-

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*Inhibition of 26 S Proteasome by Polyubiquitin Chains* 23719

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*Inhibition of 26 S Proteasome by Unanchored Polyubiquitin Chains*

Data are taken from Fig. 6. For Ub2 and Ub3, values of \( K_{0.5} \) were calculated based on the inhibition observed at a single concentration of the respective chain.
cause the high level of the proteasome in cells (~1% of cell protein (50)) means that recognition site(s) should be present at a concentration of 1–2 µM.

The K_{0.5} values measured in the current study may underestimate the true affinities of polyubiquitinated conjugates for two reasons. First, observed K_{0.5} values will exceed true K_{v} by a factor related to [S]/[K_{m}]. Although the concentration of ubiquitin-125I-lysozyme conjugates in our assays was only ~30 nM, K_{m} is unknown. In addition, conjugated fraction II proteins were present at an undetermined concentration (“Experimental Procedures”). A finding that Ub₄ inhibits the degradation of a Ub₄-RNase A conjugate with K_{0.5} ~ 10 µM suggests that the current K_{0.5} values may underestimate true affinity. However, such competition effects would not change the relative affinities of different length polyubiquitin chains (Table II).

Finally, the approach taken here should be applicable to chains linked through Lys residues other than Lys⁴⁸. If an enzyme can be identified to catalyze formation of the desired isopeptide bond, application of the methodology described here could facilitate the structural and functional characterization of such novel chains.

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REFERENCES

1. Ciechanover, A. (1994) Cell 79, 13–21
2. Hochstrasser, M. (1996) Annu. Rev. Genet. 30, 405–439
3. Rubin, D. M., and Finley, D. (1995) Curr. Biol. 5, 854–858
4. Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983) J. Biol. Chem. 258, 8206–8214
5. Scheffner, M., Nufer, U., and Huber, J. M. (1995) Nature 373, 81–83
6. Hershko, A., Leshinsky, E., Gatoff, D., and Heller, H. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1619–1623
7. Hough, R., Pratt, G., and Rechsteiner, M. (1986) J. Biol. Chem. 261, 2400–2408
8. Shaffer, J. R., and Kania, M. A. (1995) Biochemistry 34, 4015–4021
9. Hershko, A., and Heller, H. (1985) Biochem. Biophys. Res. Commun. 128, 1079–1086
10. Cha, V., Tobias, J. W., Bachmaier, A., Marriotti, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) Science 245, 1576–1583
11. Einer, T., and Ellison, M. J. (1994) Mol. Cell. Biol. 14, 7876–7883
12. Finley, D., Sadis, S., Monia, B. P., Boucher, P., Ecker, D. J., Crooke, S. T., and Cha, V. (1994) Mol. Cell. Biol. 14, 5501–5508
13. Spanel, J., Sadis, S., Haas, A. L., and Finley, D. (1995) Mol. Cell. Biol. 15, 1265–1273
14. Johnson, E. S., Ma, F. C. M., Ota, I., and Varshavsky, A. (1995) J. Biol. Chem. 270, 17442–17453
15. Baboshina, O. V., and Haas, A. L. (1996) J. Biol. Chem. 271, 2823–2831
16. Gregori, L., Pooch, M. S., Cousins, G., and Chau, V. (1990) J. Biol. Chem. 265, 8354–8357
17. Beal, R., Deveraux, Q., Xia, G., Rechsteiner, M., and Pickart, C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 861–866
18. Hoffman, L., Pratt, G., and Rechsteiner, M. (1992) J. Biol. Chem. 267, 25992–25998
19. Chu-Ping, M., Slaughter, C. A., and DeMartino, G. N. (1992) Biochem. Biophys. Acta 1119, 303–311
20. Dubiel, W., Mattel, K., and Rechsteiner, M. (1995) Mol. Cell. Biol. 15, 27–34
21. Lowe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R. (1995) Science 268, 533–539
22. Groll, M., Dietel, L., Lowe, J., Stock, D., Bohm, M., Bartunik, H. D., and Huber, R. (1997) Nature 386, 463–471
23. Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994) J. Biol. Chem. 269, 7059–7061
24. Van Noeker, S., Deveraux, Q., Xia, G., Rechsteiner, M., and Vierstra, R. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 856–860
25. DeMartino, G. N., Moodaw, C. R., Zagnitko, O. P., Prosk, R. J., Chu-Ping, M., Afonis, S. J., Swaffield, J. C., and Slaughter, C. A. (1994) J. Biol. Chem. 269, 20878–20884
26. Van Noeker, S., Sadis, S., Rubin, D. M., Glickman, M., Fu, H., Coux, O., Wefes, J., Finley, D., and Vierstra, R. D. (1996) Mol. Cell. Biol. 16, 6020–6028
27. Cox, M. J., Shapira, R., and Wilkinson, K. D. (1996) Annu. Rev. Biochem. 55, 345–352
28. Zaret, J. K., and Pickart, C. M. (1990) J. Biol. Chem. 265, 21835–21842
29. Pickart, C. M., and Vella, A. T. (1994) J. Biol. Chem. 269, 12028–12035
30. Haldeman, M. T., Xia, G., Kasperek, E. M., and Pickart, C. M. (1997) Biochemistr. 36, in press
31. Miller, H. I., Henzel, W. J., Ridgway, J. B., Kuang, W.-J., Chisholm, V., and Liu, C.-C. (1989) Bio/Technology 7, 698–704
32. Mayer, A. N., and Wilkinson, K. D. (1989) Biochemistry 28, 166–172
33. Duxten, R. L., and Cohen, R. E. (1989) J. Biol. Chem. 264, 16739–16747
34. Weber, J. D., Butt, T. R., Marsh, J. S., Sternberg, E. J., Margolis, N., Moon, B. P., Jannalagadda, S., Khan, M. I., Weber, P. L., Mueller, L., and Crooke, S. T. (1987) J. Biol. Chem. 262, 14213–14221
35. Burch, T. J., and Haas, A. L. (1998) Biochemistry 37, 7300–7308
36. Haas, A. L., and Wilkinson, K. D. (1985) Prep. Biochem. 15, 49–60
37. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77
38. Pickart, C. M., Halldenman, M. T., Kasperek, E. M., and Chen, Z. (1992) J. Biol. Chem. 267, 14418–14223
39. Cole, R. D. (1967) Methods Enzymol. 11, 315–317
40. Chen, Z., Niles, E. G., and Pickart, C. M. (1991) J. Biol. Chem. 266, 15698–15704

*W. Xu and R. Cohen, unpublished experiments.*
41. van Nocker, S., and Vierstra, R. D. (1993) *J. Biol. Chem.* **268**, 24766–24773
42. Cox, M. J., Haas, A. L., and Wilkinson, K. D. (1986) *Arch. Biochem. Biophys.* **250**, 400–409
43. Herskko, A., Ganoth, D., Pehrson, J., Palazzo, R. E., and Cohen, L. H. (1991) *J. Biol. Chem.* **266**, 16376–16379
44. Dunten, R. L., Cohen, R. E., Gregori, L., and Chau, V. (1991) *J. Biol. Chem.* **266**, 3260–3267
45. Lam, Y. A., Xu, W., DeMartino, G. N., and Cohen, R. E. (1997) *Nature* **385**, 737–740
46. Shaeffer, J. H. (1994) *J. Biol. Chem.* **269**, 29530–29536
47. Finley, D., Bartel, B., and Varshavsky, A. (1989) *Nature* **338**, 394–401
48. Cook, W. J., Jeffrey, L. C., Kasperek, E. M., and Pickart, C. M. (1994) *J. Mol. Biol.* **236**, 601–609
49. Haas, A. L., and Bright, P. M. (1985) *J. Biol. Chem.* **260**, 12464–12473
50. Tanaka, K., Hi, K., Ichihara, A., Waxman, L., and Goldberg, A. L. (1986) *J. Biol. Chem.* **261**, 15197–15203
51. Pickart, C. M., and Rose, I. A. (1985) *J. Biol. Chem.* **260**, 1573–1581
52. Wefes, I., Kaiser, P., Schneider, R., Pickart, C. M., and Finley, D. (1995) *Gene (Amst.)* **163**, 321–322
53. Haas, A., Reback, P. M., Pratt, G., and Rechsteiner, M. (1990) *J. Biol. Chem.* **265**, 21664–21669
54. Johnson, E. S., Bartel, B., Seufert, W., and Varshavsky, A. (1992) *EMBO J.* **11**, 487–505
55. Johnson, E. S., Ma, P. C. M., Ota, I. M., and Varshavsky, A. (1995) *J. Biol. Chem.* **270**, 17442–17456
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