A 25-Kilodalton Ubiquitin Carrier Protein (E2) Catalyzes Multi-ubiquitin Chain Synthesis via Lysine 48 of Ubiquitin

Zhijian Chen and Cecile M. Pickart†

From the Department of Biochemistry, State University of New York, Buffalo, New York 14214

Target protein multi-ubiquitination involving lysine 48 of ubiquitin (Ub) is known to occur during protein degradation in the ATP- and Ub-dependent proteolytic pathway (Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) Science 243, 1576–1583). However, little is known about the enzymatic mechanism of multi-ubiquitination. We show that a purified Ub carrier protein, E225K, catalyzes multi-Ub chain synthesis from purified Ub. Incubation of E225K with Ub activating enzyme (E1), MgATP, and radiolabeled Ub (M, = 8500) resulted in time-dependent appearance of a “ladder” of radiolabeled Ub conjugates with molecular masses of 8.5k kDa, where n = 1, 2, 3, 4… (up to at least n = 10). The kinetics of this conjugative process were consistent with Ub2 acting as a steady-state intermediate. The putative Ub2 product of E225K catalysis was purified and cleaved with a partially purified isopeptidase preparation. The sole cleavage product (M, = 8500) had a tryptic digest identical to that of authentic Ub, confirming that the original conjugate was Ub2. Tryptic digestion of intact Ub2 gave products consistent with the existence of an isopeptide linkage between the COOH terminus of one Ub and Lys-48 of the other; this structure was confirmed by sequence analysis of the unique Ub2 tryptic fragment. Tryptic digestion of higher order Ub, adducts (n ≥ 4) yielded fragments identical to those of Ub2, indicating that E225K ligates successive Ub molecules primarily or exclusively via Lys-48. Although several other E2s supported synthesis of an apparent Ub, adduct of undetermined linkage, only E225K was capable of synthesizing multi-Ub chains from isolated Ub.

Quantitative analysis of single turnovers showed that transfer from E225K-Ub to Ub and Ub2 occurred with k2 = 488 and 1170 m-1 min-1, respectively, at pH 7.3 and 37 °C. These results show that increasing the number of Ub molecules in a chain increases susceptibility to further ubiquitination by E225K. Ub2 was a good substrate for activation by E1 and was readily transferred to E225K. The labile E225K-Ub2 adduct was catalytically active, and exhibited preference for Ub2 (versus Ub1) as acceptor. These results suggest that E225K may function as a multi-ubiquitinating enzyme in the Ub-dependent proteolytic pathway.

Eukaryotic cells possess a complex enzymatic pathway devoted to ligation of the small, highly conserved protein Ub to other cellular proteins (1, 2). Ubiquitination apparently mediates a variety of cellular functions, the best understood of which is specific proteolysis. In the Ub-dependent proteolytic pathway, ubiquitination renders cellular proteins susceptible to degradation by a specific protease (1).

The formation of a degradative Ub conjugates occurs in three steps (3). Initially the Ub carboxyl terminus undergoes ATP-dependent activation, resulting in formation of a Ub thiol ester with Ub activating enzyme or E1. Ub is then transferred to a thiol group of a Ub carrier protein or E2. Finally a ligase, E3, catalyzes transfer of Ub from the E2 to an ω-amino group of a target protein. Recent studies suggest that conjugate-multi-ubiquitination may be important in degradation (4, 5). Multi-ubiquitinated β-galactosidase conjugates have structures in which there is only one Ub-β-galactosidase bond; other Ub molecules are ligated to Lys-48 residues of Ub (4, 5). The mechanism of formation of such multi-ubiquitinated conjugates is not currently understood.

Based on their abilities to form labile Ub adducts, at least seven E2s exist in rabbit reticulocytes (6–9), and in the yeast Saccharomyces cerevisiae (10). In reticulocytes, only E214K is known to function with E3 in the proteolytic pathway (6, 7); in S. cerevisiae, a pair of closely related 16-kDa E2s may carry out a similar function (11). Reticulocyte E214K, E220K, E225K, and E226K all exhibit E3-independent ubiquitinating activity in vitro, suggesting that they may function in Ub conjugate-mediated regulation of biological processes (6, 9, 11–13). Similar suggestions have been made for the mechanisms by which two yeast E2s function in DNA repair and progression of the cell cycle (10, 14, 15). There are also E2s, e.g. E217K and E220K from reticulocytes, for which no conjugative activity has yet been demonstrated (7, 8, 12, 13).

The results reported here originated from a search for E225K conjugative substrates. In experiments in which a high concentration of unlabeled Ub was added to “chase” labeled Ub from the E2 adduct into a putative substrate, we observed a prominent conjugate of the size expected for Ub5. As reported here, studies on this latter reaction show that E225K multi-ubiquinates Ub itself; that Ub5 chains have branched structures in which an isopeptide bond links the COOH-terminal Gly residue of Ub5 and the ω-amino group of Lys-48 of Ub5-1; and that Ub5 is itself a substrate for E225K-catalyzed activation and transfer. These results apparently account for the recently described formation of “diubiquitin” in reticulo-
cyte extracts incubated with high concentrations of des-GlyGly-Ub (16).

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise indicated, all reagents and proteins were obtained from Sigma. [γ-32P]ATP (3000 Ci/mmol) was from Du Pont-New England Nuclear. Des-GlyGly-Ub was pre-scrubbed previously (17), and partially purified by gel filtration (FPLC Superose 12 column, Pharmacia Biotechnology Inc.). Quantitative cleavage was confirmed by the inability of des-GlyGly-Ub to support Ub-dependent proteolysis. Recticulate E1, various recticulate E2s, and Ub were purified to electrophoretic homogeneity as described previously (17).

**Enzyme Assays**—Unless indicated otherwise, all assays were conducted at pH 7.3 and 37°C, under the following conditions: 50 mM Tris-HCl (20% base), 5 mM MgCl2, 2 mM ATP, 10 mM phosphocreatine, and 0.6 unit/ml each of inorganic pyrophosphatase and creatine kinase. Protein concentrations were determined using Bio-Rad dye reagent, with BSA as standard. Active E1 and E2 concentrations were obtained using [γ-32P]ATP in the PPi burst assay described previously (6). This assay was also used to monitor ubiquitination of the buffer contained 0.1 M KCl, and 0.1 mM EDTA, and 1 mg/ml ovalbumin, and 5 μM Ub. (745 cpm/pmol).

Incubations were quenched after 5 min with 10 μl of SDS-PAGE sample buffer and heated to 100°C prior to analysis by SDS-PAGE and autoradiography.

**Large Scale Synthesis and Purification of Ub**—Ubications were performed in a 97°C column, Pharmacia Biotechnology Inc.; quantitative cleavage was confirmed by the inability of des-GlyGly-Ub to support Ub-dependent proteolysis. Recticulate E1, various recticulate E2s, and Ub were purified to electrophoretic homogeneity as described previously (17).

**Results**—The unique Ubz peptide b, which eluted at 15.5 min, was collected and concentrated (to 500 μl) using a Millipore concentrator. Digestion was judged to be essentially complete based on the disappearance of the reverse-phase peak corresponding to intact Ub. Digestion of Ub(Fab2)I (initially 1.2 mg/ml) was done similarly. Tryptic peptides eluted at 0.5 min. The eluate was concentrated (to 0.5 ml) using a Millipore concentrator. Final concentrations of Ub and Ubz were calculated from the absorbance at 205 nm and absorbance was recorded on a computing integrator (Milan Roy CI4000; attenuation setting = 6).

**Isolation of Altered Tryptic Peptide—**Ubz (15 μl, 10 mg/ml) was digested with trypsin in a final volume of 30 μl (above) and subjected to reverse-phase HPLC as above, except that the following elution protocol was used: 0 min, 55% B; 5 min, 55% B; 50 min, 75% B; 52.5 min, 75% B; 70 min, 100% B. Peptides were isolated using a C18 column (1.9 mm x 125 mm). Peptides were separated by reverse-phase HPLC as above, except that the following elution protocol was used: 0 min, 55% B; 5 min, 55% B; 50 min, 75% B; 52.5 min, 75% B; 70 min, 100% B. (Buffers A and B were those of Ref. 23.) Peptides T1 through T7 eluted in the void volume. Other peptides eluted at 9.5 (T8), 12 (T9), 17 (T10), and 19.5 min (T11). The unique Ubz peptide b, which eluted at 15.5 min, was collected (see "Results").

**RESULTS**

Purification of E2-25K from Calf Thymus As shown in Fig. 1, lane 3, the DTT eluate obtained from calf thymus fraction II by covalent Ub affinity chromatography (3) contained E1, E2s, and several 14-kDa E2 isoforms (below). E2sK and E2sK, which are abundant in DTT eluates prepared from reticulocyte fraction II (6-8), were absent. Chromatography of the DTT eluate on an FPLC Mono Q anion exchange column yielded electrophoretically homogeneous E2sK (Fig. 1, lane 4), typically about 180 μg from 300 g of thymus. Significant amounts of E2sK (60 μg), of E2sK (20 μg), and of a previously undescribed 14-kDa E2 were also recovered. As detected in assays of labile Ub adduct
Fig. 1. Purification of $E_{25K}$ from calf thymus. A 12.5% gel (without urea) was stained with Coomassie Blue. Lane 1, calf thymus fraction II (50 µg of protein); lane 2, flow-through of Ub-Sepharose column (81 µg); lane 3, concentrated DTT eluate from Ub-Sepharose column (5 µg). The marker proteins were: BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and yeast cytochrome c (12 kDa). The 30-kDa protein in lane 3 did not form a labile Ub adduct. The high molecular weight bands in lane 3 are E1 (~100 kDa) and BSA (carrier, 68 kDa).

Fig. 2. Copurification of $E_{25K}$ and Ub-$\alpha$-forming activity. Assays were performed using fractions from the FPLC Mono Q column (text), and were quenched with an equal volume of thiol ester prior to electrophoresis (not shown). If an aliquot (15 µl) was then analyzed by electrophoresis on SDS gels followed by staining, drying, and autoradiography, Lanes 1 and 6, fraction 17; lanes 2 and 7; fraction 18; lanes 3 and 8, fraction 19; lanes 4 and 9, fraction 20; lanes 5 and 16, fraction 21. A, autodigraph of labile Ub adduct assays; 2 µl of a 5-fold diluted aliquot was used for each assay (10 µl) which also contained 2 µM $^{35}$S-Ub (15,000 cpm/µmol) and E1 (80 nM). Incubations were for 3 min. B, autoradiograph of Ub synthesis assays. Ub adducts were formed as in A. After 3 min, 1 µl of unlabeled Ub was added to give a final concentration of 16 µM. Incubation was continued for 60 min at 37°C. C, quantitation of data in A and B by excision and counting of the relevant bands: $E_{25K}$-Ub adduct (x); Ub$(\square)$; Ub adduct of 14-kDa E2 described in text (O).

formation, the latter species eluted from the Mono Q column just ahead of $E_{25K}$ (lanes 2 and 3, Fig. 2A). This was significantly later than the elution of the $E_{25K}$ (7) recovered from the same column (not shown). We do not know whether this 14-kDa species (Fig. 2A) represents a new E2 isoform, or a degradation product (e.g. of $E_{25K}$). A small amount of $E_{25K}$, which elutes much later from Mono Q (7, 8), was also recovered (not shown).

Calf thymus $E_{25K}$ was indistinguishable from reticulocyte $E_{25K}$ based on the following criteria (6, 7). (a) Both migrated on SDS-PAGE at 25 kDa (Fig. 1). (b) Reticulocyte and thymus proteins eluted identically from a Mono Q column (see "Experimental Procedures"). (c) Both catalyzed Ub transfer to free lysine (50 mM) with a rate of 0.15 pmol of Ub/pmol of E2/min (not shown). (d) Both were essentially inactive in histone ubiquitination (not shown).
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E2\textsubscript{AUK}-catalyzed Multi-ubiquitination

Fig. 3. Time course of E2\textsubscript{AUK}-dependent multi-Ub chain synthesis from Ub (A–C) and Ub\textsubscript{2} (C). For A and B, incubations contained [\textsuperscript{35}S]-Ub (1000 cpm/pmol), E2\textsubscript{AUK} (0.64 \mu M), and E1 (80 nM). Aliquots of 10 \mu l were withdrawn at increasing times, quenched with 10 \mu l of normal sample buffer which contains mercaptoethanol, and heated to 100 °C prior to electrophoresis of 1.5-\mu l aliquots. A, autoradiograph. Lane 1, 0 min; lane 2, 10 min; lane 3, 20 min; lane 4, 40 min; lane 5, 1 h; lane 6, 2 h; lane 7, 4 h; lane 8, 6 h; lane 9, 8 h. B, quantitation (by excision and counting of relevant bands): Ub, (0); radiograph: multi-Ub chain synthesis from Ub (\textsubscript{A-C}) and Ubn (C). For A and B, incubations with the same isopeptidase preparation gave substantial apparent half-lives (above). C, autoradiograph: multi-Ub chain synthesis from Ub (lanes 1–4) versus Ubn (lanes 5–8). Incubations contained 94 nM E1, 0.64 \mu M E2\textsubscript{AUK}, and 12 \mu M Ub or Ub\textsubscript{2} (980 cpm/pmol in each case). Aliquots (10 \mu l) were withdrawn at increasing times and quenched as above, followed by electrophoresis and autoradiography. Lanes 1 and 5, 0 min; lanes 2 and 6, 1 h; lanes 3 and 7, 2 h; lanes 4 and 8, 4 h. The Ub\textsubscript{2} preparation was slightly contaminated with Ub (text). The numbers on the right are the n values for the indicated Ub products.

a product which appeared to be Ub, based on its molecular mass of 8.5 kDa (Fig. 4A, lane 1). Treatment of Ub\textsubscript{2} with the same isopeptidase preparation gave substantial apparent conversion to Ub, although some Ub oligomers were resistant to disassembly (Fig. 4A, lane 3). These results are consistent with the structures (Ub\textsubscript{2} and Ub\textsubscript{2+4}) assigned to these conjugates (above).

The major proteins in the crude isopeptidase preparation used in Fig. 4A were separated by FPLC gel filtration chromatography, and the activity of each in Ub\textsubscript{2} cleavage was examined (see “Experimental Procedures”). Present at \sim 10 \mu g/ml, the 98-kDa protein and UCH each completely cleaved 5 \mu M Ub\textsubscript{2} (to Ub) within 30 min. Under more stringent conditions the 98-kDa protein exhibited a higher specific activity: with Ub\textsubscript{2} at \sim 2 \mu g/ml, there was barely detectable cleavage of 5 \mu M Ub\textsubscript{2} in 5 min; with the 98-kDa protein at 2 \mu g/ml, there was more than 50% cleavage in 5 min (not shown). These qualitative results suggest that the 98-kDa protein is an isopeptidase which exhibits higher activity toward Ub-Ub linkages than does UCH.

Fig. 4. Identification of the site of isopeptid linkage in Ub, and Multi-Ub Chains—The isopeptidase-treated samples served as controls in experiments designed to characterize the Ub-Ub linkages in E2\textsubscript{AUK} catalytic products. We thought it likely that these linkages would consist of isopeptide bonds between Ub COOH termini and one or more Ub Lys residues. In this case, comparison of the tryptic maps for free Ub and the conjugates should allow deduction of the linkage(s) (4), since Lys residues involved in isopeptide bonds are resistant to tryptic cleavage.

Reverse-phase HPLC analysis of the tryptic digest of the sample in lane 1 (Fig. 4A) is shown in panel i (Fig. 4). The profile is essentially identical to that reported previously for authentic Ub (23), and to that obtained by us for Ub\textsubscript{1} (unmodified Ub which went through the same manipulations as Ub\textsubscript{2}; see “Experimental Procedures”). The result shown in panel i demonstrates that, within our detection limits, putative Ub\textsubscript{2} (Fig. 4A, lane 2) is indeed composed solely of Ub.

The digest of intact Ub\textsubscript{2} is shown in panel ii (Fig. 4). Two differences distinguish this profile from that of the control (panel i). First, the sizes of the peaks for peptides T4 (residues 49–54) and T8 (residues 43–48) are about 2-fold reduced in ii relative to i. This is the result expected for isopeptic linkage via Lys-48, since only one of the Ub molecules in the dimer...
is blocked at Lys-48 (Scheme I).  

Leu-73  Arg-74  Gly-75  Gly-76  T2
Leu Ile Phe Ala Gly Lys Gln Leu Gln Asp Gly Arg
43 44 45 46 47 48 49 50 51 52 53 54
T8  T4

SCHEME I

The Ub digest also differs from the Ub digest in that there are two unique peaks, designated a and b, in the Ub profile (panel ii). Analysis of the time course of tryptic digestion (not shown) indicated that peptide a was a precursor of peptide b. Peptide b was isolated (see “Experimental Procedures”) and sequenced; the results (Table I), together with those shown in Fig. 4, establish unequivocally that Lys-48 is the specific and exclusive residue at which isopeptic linkage occurs in Ub synthesized by E2_{25K} (Scheme I). Given the structure of peptide b, its slow production from peptide a (above) most likely reflects slowing of tryptic cleavage at Arg-74 due to steric hindrance from the isopeptide linkage at Gly-76 (23).

The results shown in panels iii and iv (Fig. 4) suggest that the Ub units in multi-Ub chains (n ≥ 4) are also linked through Lys-48. The tryptic digest of the product of isopeptide digestion of Ub_{nax} (panel iii) was essentially identical to that of Ub (compare with panel i), indicating that Ub is the only detectable constituent of products apparently containing up to 10 Ub molecules. The tryptic digest of intact Ub_{nax} (panel iv) shows strongly reduced amounts of peptides T4, T8, and T2 (residues 73-76), together with the altered peptides (a and b) seen in the Ub profile (compare with panel ii). Again this is the expected result if the major linkage in these products is through Lys-48; as n becomes large, most of the Ub units in the chain will be blocked at Lys-48, causing yields of T2, T4, and T8 to be highly attenuated (Scheme I). Because of the HPLC profile of Ub_{nax} is noisier than that of Ub (due to the smaller amount of Ub_{nax}), it is possible that a minor fraction of the Ub-Ub linkages in Ub_{nax} products are not through Lys-48. However, there is no actual positive evidence in support of this hypothesis. We conclude that multi-Ub chain synthesis by E2_{25K} occurs largely, possibly exclusively, through linkage of Ub COOH termini to Lys-48.

Ub is a Competent Acceptor for Ubiquitination Catalyzed by E2_{25K}—We wished to demonstrate the competency of Ub_{2} as an intermediate in multi-Ub chain synthesis catalyzed by E2_{25K} (cf. Fig. 3B). For this purpose, E2_{25K}-Ub was formed in a brief incubation with a low concentration of highly labeled 125I-Ub (together with E1 and MgATP); EDTA was added to chelate Mg^{2+} and prevent further adduct formation; low specific radioactivity Ub or Ub_{2} (12.2 μM in each case) was then added to act as acceptor. The disappearance of E2_{25K}-Ub and the appearance of Ub or Ub_{2} were monitored by SDS-PAGE.

E2_{25K}-Ub disappearance occurred concomitant with formation of the expected product (Ub, in Fig. 5A; Ub_{2} in Fig. 5B). Product formation was pseudo first-order in each case.
(Fig. 6A), but the rate constant with Ub2 as acceptor (0.84 h⁻¹; filled circles) was 2.5 times larger than that with Ub (0.36 h⁻¹; open circles). Note that this difference is not a simple concentration effect, while Ub2 consists of two Ub molecules, each mol of Ub2 contains only 1 mol of the relevant acceptor residue, Lys-48. In other single-turnover experiments, the rate of Ub2 or Ub2 formation was evaluated as a function of acceptor concentration. The dependence was essentially linear in each case (Fig. 6B), with second-order rate constant for Ub2 (0.014 min⁻¹/1.2 × 10⁻⁵ M = 1170 M⁻¹ min⁻¹) being 2.4 times larger than that for Ub. Thus Ub2 is not only competent to act as an acceptor for Ub transfer from E2₂五百-K-Ub, but it is a better acceptor than the Ub monomer.

Ub2 is a Competent Substrate for Activation and Transfer from E1 to E2五百-K—Might Ub2 itself be activated and transferred? We addressed the first part of this question by testing the ability of Ub2 to support PP formation from [γ-³²P]ATP in the presence of E1 and Mg²⁺ (25). Upon addition of E1 (1.5 pmol = 78 nM) to an incubation containing 7.8 µM Ub, there was rapid production of 2 mol of [³²P]PP/mol E1, corresponding to the expected formation of the E1-Ub·AMP·Ub aduct (Fig. 7A). The formation of an E1-Ub₂ adduct was inferred from the kinetic rate that is indistinguishable (in this assay) from those in E1-Ub formation under these conditions. Competition studies (Fig. 7B) suggest that Ub and Ub₂ bind similarly to E1: when E1 was added to incubations containing radiolabeled Ub (at constant concentration) and increasing concentrations of unlabeled competitor, Ub (open circles) and Ub₂ (filled circles) competed identically (Kᵣ = 5 µM). It was expected that Kᵣ for Ub would be somewhat larger than the concentration of ³²P-I-Ub in this experiment (1.3 µM), since radiolabeled Ub binds more tightly to E1 than does unmodified Ub (26).

In PP burst assays, inclusion of E2五百-K (6 pmol = 0.19 µM) caused a stoichiometric increase in burst size, independent of whether the substrate was Ub or Ub₂ (squares, Fig. 7A). Therefore Ub₂ can be transferred from E1 to E2五百-K, with kinetics that are indistinguishable (in this assay) from those for Ub. Formation of an E2五百-K-Ub₂ adduct was verified by electrophoresis analysis (see below, Fig. 5, C and D). For Ub as substrate, the steady-state rate of ATP hydrolysis was nearly independent of the presence of E2五百-K (open squares versus circles, Fig. 7A), consistent with the low nucleophilic reactivity previously reported for the E2五百-K-Ub adduct (8). However, the steady-state rate was significantly faster with Ub₂ (filled squares), indicating that the E2五百-K-Ub₂ adduct turns over faster than the E2五百-K-Ub adduct. This faster turnover is not due to E2-catalyzed Ub₂ transfer to Ub (below), since it was independent of a 2-fold increase in [Ub₂] (not shown). Therefore the labile Ub₂ adduct of E2五百-K is substantially more reactive with either water or DTT (~0.1 min⁻¹ in these assays) than is the labile E2五百-K-Ub adduct. The rate constants for E2 adduct turnover derived from Fig. 7A (after correction for the contribution of E1) were 0.01 min⁻¹ (Ub) and 0.07 min⁻¹ (Ub₂). In contrast, transfer to free lysine was less efficient with the Ub₂ adduct (0.05 versus 0.18 pmol/min/pmol E2; not shown).

The results of single-turnover experiments demonstrated that Ub₂ is transferred from E2五百-K-Ub₂ to E2五百-K (Fig. 5C) or to Ub (Fig. 5D), to form Ub or Ub₂, respectively. Quantitative analysis of these data was not possible because of 1) more rapid turnover of E2五百-K-Ub₂ (above); and 2) formation of higher-order products even during one turnover in the presence of a high concentration of the “desired” acceptor. Thus Ub₂ formation during Ub₂ transfer to “Ub” (arrowhead, Fig. 5C) is presumed to occur by successful competition of residual Ub₂ from the pulse (≤0.2 µM) with the added acceptor, Ub (12.2 µM). Similarly, the band running above the E2五百-K-Ub₂ adduct in Fig. 5D may represent Ub₂ formed by competition of the initial transfer product, Ub₂ (≤0.2 µM), with the added acceptor, Ub (12.2 µM). Note that the intensities of the bands corresponding to these unexpected products are not directly proportional to their molar concentrations, because of the low specific radioactivity of the added acceptor relative to that of the Ub₂ in the pulse (legend, Fig. 5). For instance, the specific radioactivity of the Ub₂ seen in Fig. 5C would be twice that of Ub₂ if Ub₂ arises by the mechanism suggested above.
Despite these problems, it is clear that Ub2 transfer to Ub2 (to form Ub3, Fig. 5D) is more efficient than transfer to Ub (to form Ub2, Fig. 5C). Thus preference for Ub2 as acceptor is maintained when Ub1 is the species being transferred. As excepted from the results summarized in Fig. 5, E225K is capable of multi-Ub chain synthesis when Ub2 is the predominant species available to act as substrate and acceptor (Fig. 3C). Under these conditions the expected intermediates accumulate (Ub1, Ub2, Ub3, lanes 5–8, Fig. 5C). A slight amount of Ub1 (lanes 7 and 8) reflects a slight contamination of Ub in the starting Ub2. Large products (Ub2k) accumulate in the Ub2 incubation at least as rapidly as in a control incubation containing only the Ub monomer at zero time. This is apparent from comparison of lanes 7 (Ub1) and 3 (Ub), after taking into account that for a given value of n, the specific radioactivity of Ub2 formed from Ub2 will be one-half that of Ub formed from Ub. This is because the (molar) specific radioactivities of the starting Ub and Ub2 are equal (legend, Fig. 3C).

Mechanism of E225k-catalyzed Multi-ubiquitination—E225K ubiquitinates Ub with a second-order rate constant, 488 M⁻¹ min⁻¹, which is comparable to k_cat/K_m values reported for ubiquitination of non-Ub target proteins by other E2a (6, 12, 13), and is 163 times larger than k2 for E225k-catalyzed ubiquitination of free lysine (0.15 min⁻¹/0.05 M = 3 M⁻¹ min⁻¹). The Ub units in the Ub2 and multi-Ub products of E225k catalysis are linked specifically by an isopeptide bond involving the epsilon amino group of Lys-48 of one Ub, and the COOH-terminal Gly residue of the next Ub (Fig. 4 and Table 1). However, Lys-48 is not the sole determinant of specificity in this reaction, since Ub and Ub2 each contain a single (unblocked) Lys-48, but k2 for transfer to Ub2 is 2.4 times larger than k2 for transfer to Ub (Fig. 6B).

There are at least two possible mechanisms for E225K-catalyzed multi-Ub chain synthesis. One mechanism involves transfer of Ub monomers to a growing chain. We have shown that this mechanism operates (Fig. 5, A and B), and that there is preference for Ub oligomers as acceptors (above, Fig. 6B). Thus formation of larger adducts will be kinetically favored as oligomers build up and the concentration of free Ub decreases. It is possible that transfer rate constants continue to increase with the size of the acceptor (cf. Fig. 6B), but experiments to measure these rate constants are unfortunately precluded by our current inability to obtain homogeneous preparations of oligomers of n > 2.

A different multi-ubiquitination mechanism involves addi-
radiation (4, 5). Our results (Fig. 3 and other data) show that E225K uniquely catalyzes synthesis of Lys-48-linked multi-Ub chains from isolated Ub, independent of the presence of any other Ub pathway component. Based on this observation and those of others (4, 5), we hypothesize that E225K functions physiologically in Ub-dependent protein degradation. One possibility is that E225K acts on proteins to which one or a few Ub molecules have already been ligated through the actions of other pathway components (e.g., E214K and E3). This kind of role, in which E225K acts only to ubiquitinate Ub, would be consistent with the results reported here and with the failure of E225K to ubiquitinate other target proteins tested to date. However, it remains possible that E225K is active in de novo multi-ubiquitination of as yet unidentified target proteins, either alone or in combination with an E3. E225K is one of the most abundant E2s in immature and mature erythroid cells (7, 29), in a number of other rabbit tissues, and in bovine thymus (see “Results”). This apparently wide distribution would be consistent with a general role for E225K in the Ub-dependent proteolytic pathway.

While the results of reconstitution studies (6, 7) suggest that E224K (with E3) is sufficient to support the formation of degradable conjugates, these results do not exclude a role for E225K in degradation. First, it is not known that Lys-48-linked multi-ubiquitinated conjugates were actually formed in the reconstitution assays, since alternative modes of ubiquitination of mono-ubiquitinated conjugate predominates during degradation (4, 5). Our results (Fig. 3 and other data) show that the two E2s also differ in their substrate specificities in multi-Ub chain formation. While E225K multi-ubiquitinates isolated Ub, E225K apparently prefers to multi-ubiquitinate Ub which is ligated to another protein.

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1. Pickart, C., Graziani, L., and Finley, unpublished experiments by the methods described in Ref. 20. Soluble extracts from rabbit liver, brain, and muscle were surveyed.

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