Polyubiquitin (Ub) chains linked through Lys-48–Gly-76 isopeptide bonds represent the principal signal by which substrates of the Ub-dependent protein degradation pathway are targeted to the 26 S proteasome, but the mechanism(s) whereby these chains are assembled on substrate proteins is poorly understood. Nor have assembly mechanisms or definitive functions been assigned to polyubiquitin chains linked through several other lysine residues of ubiquitin. We show that rabbit reticulocyte lysate harbors enzymatic components that catalyze the assembly of unanchored Lys-29-linked polyubiquitin chains. This reaction can be reconstituted using the ubiquitin-conjugating enzyme (E2) known as UbcH5A, a 120-kDa protein(s) that behaves as a ubiquitin-protein ligase (E3), and ubiquitin-activating enzyme (E1). The same partially purified E3 preparation also catalyzes the assembly of unanchored chains linked through Lys-48. Kinetic studies revealed a $K_m$ of $-9 \mu M$ for the acceptor ubiquitin in the synthesis of diubiquitin; this value is similar to the concentration of free ubiquitin in most cells. Similar kinetic behavior was observed for conjugation to Lys-48 versus Lys-29 and for conjugation to tetraubiquitin versus monoubiquitin. The properties of these enzymes suggest that there may be distinct pathways for ubiquitin-ubiquitin ligation versus substrate-ubiquitin ligation in vivo.

The Ub-dependent proteolytic pathway is the principal mechanism for turnover of short-lived proteins in eukaryotic cells (1, 2). By helping to set the levels of key intracellular regulatory proteins, the Ub pathway plays a major role in critical processes such as the regulation of the cell cycle (reviewed in Ref. 3). The function of Ub in proteolysis is that of a covalent signal; attachment of the C terminus of Ub (G76) to the side chain of an internal lysine residue of the target protein ultimately confers recognition by the 26 S proteasome. Conjugation occurs in three sequential enzymatic steps (3): 1) ATP-dependent activation of the Ub C terminus through the formation of a thiol ester with Ub-activating enzyme or E1; 2) Ub transfer to a Cys residue of a Ub-conjugating enzyme or E2; and 3) Ub transfer from the E2 to the substrate catalyzed by a ligase or E3. Specific E2s and E3s cooperate in the recognition of individual substrates of the pathway (3). These two enzymes form a complex; the E3 appears to play the major role in substrate binding.

Substrates destined for turnover by the proteasome are usually conjugated to multiple Ub molecules in the form of a polymeric chain linked by isopeptide bonds between Lys-48 of Ub$_n$, and Gly-76 of Ub$_{n+1}$ (4–6). The extreme inhibition of proteolysis caused by the K48R mutation in Ub indicates that targeting to the proteasome is mediated primarily through the recognition of Lys-48-linked polyUb chains (4, 5, 7). We have presented evidence that the proteasomal signaling function of these chains depends upon a specific conformation that is stabilized by defined contacts among the Ub moieties in the chain (8–11).

Little is yet known about the enzymatic mechanisms by which chains are assembled on target proteins (see Ref. 6). One possibility is that the same E3 that conjugates the first Ub to the substrate also elongates the chain. An alternative model postulates the existence of two types of conjugating pathways, namely substrate-specific and Ub-specific. A Ub-specific E2/E3 could preassemble a chain for use by a substrate-specific E2/E3, or a Ub-specific E2/E3 could extend a chain from an initiator Ub conjugated to the target protein by a substrate-specific E2/E3 (see “Results and Discussion”). Although there are detectable levels of unanchored polyUb chains in cells (7, 12–14), it is not known whether these species arise by de novo synthesis from free Ub or as by-products of conjugate turnover (15, 16). However, support for the existence of Ub-specific conjugating enzymes derives from the properties of the UFD2 and UFD4 gene products of Saccharomyces cerevisiae (Refs. 17 and 18).

PolyUb chains linked through lysine residues other than Lys-48 have been identified both in cells and in vitro (reviewed in Ref. 19). The degradation of a Ub-DHFR fusion protein in yeast cells depends on the ligation of a polyUb chain to Lys-29 of the fused Ub moiety and on the presence of an E3, Ufd4p, which appears to recognize this Ub moiety (17). In some instances, the Ufd4p-dependent elongation of polyUb chains also requires a novel factor known as Ufd2p (17, 18). In such cases, short Ub oligomers assembled by Ufd4p initiate at Lys-29, but the subsequent linkages in these short chains and in longer chains assembled in the presence of Ufd2p are uncharacterized (18). Several other lysine residues of Ub have also been implicated in polyUb chain assembly. Purified human E2$_{HBP}$ cooperates with an unidentified E3 to assemble chains harboring Lys-11 linkages (20); these chains are functional in degradation in vitro (21). The proteolytic signaling competence of chains harboring Lys-6 linkages, which can be assembled on histones by purified Rad6p, remains to be evaluated (20). Chains harboring Lys-63 linkages apparently do not play an important role in protein turnover, but they have been implicated in DNA.
repair (14, 22), the stress response (23), and endocytic signaling (24) in yeast. Thus, Lys-63-linked chains and perhaps certain other chains may signal fates other than proteasomal proteolysis.

We are interested in the signaling potentials of novel polyUb chains and in the identities and properties of enzymes that assemble such chains in vivo. The studies described here were motivated by results of Bamezai and Breslow (25), who reported the assembly of unanchored polyUb chains in a manner that depended on at least two factors in reticulocyte lysate (besides E1). We developed an assay for the synthesis of novel chains and applied it to characterize a specific E2 and an apparent E3(s), which cooperate in the assembly of Lys-29- and Lys-48-linked chains. Their properties suggest that these enzymes are likely to function in chain assembly in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods**—Reagents and proteins were from Sigma unless stated otherwise. Wild-type (Sigma) or K48R-Ub was radiolabeled to ~8,900 cpm/μmol with [35S]methionine and carrier ubiquitin-T (26). E1 was purified from bovine erythrocytes (27). UbA was provided by K. Wilkinson (Emory University) or R. Cohen (University of Iowa) (28, 29). Rabbit reticulocytes were purchased from Green Hectares (Oregon, WI). Reticulocyte lysate, fraction I, and fraction II were prepared as described (26). SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (30).

**Mutant and Chemically Modified Ubs**—The expression and purification of recombinant K48R-Ub and K48C-Ub have been described (10). Purified wild-type Ub, and K48R-Ub, generated through the expression of recombinant K48R-Ub and K48C-Ub have been described (10).

**Purified UbcH5A.**—A rabbit E2 preparation enriched for Ubc5 was prepared as follows. Proteins precipitating between 0 and 55% saturation with ammonium sulfate were collected from rabbit reticulocyte fraction I, dialyzed against MonoQ buffer (above), and applied to an S-Sepharose column pre-equilibrated with MonoQ buffer. The flow-through was discarded, and the column was eluted with three column volumes of MonoQ buffer containing 0.5 M NaCl. S-Sepharose chromatography was necessary to remove Ub, which would otherwise interfere in the assay step. Proteins in the eluate were precipitated with 80% ammonium sulfate and dialyzed against MonoQ buffer. The dialyzed eluate was supplemented with purified E1 (50 nm) and MgATP and chromatographed on a Ub-Sepharose column (26). Covalently bound species were eluted with MonoQ buffer containing 5 mM DTT and 0.1 mg/ml ovalbumin. The eluate was concentrated by ultrafiltration. The eluate contained a prominent protein of ~16 kDa (presumptive Ubc5; see “Results”), which formed a thiorester with Ub as well as a minor thiol ester-forming protein of a slightly higher molecular weight (not shown). The concentration of the presumptive Ubc5 was estimated based on the concentration of the 24-kDa I2-1-Ub thiol ester band, as determined by comparison to the result obtained with a known amount of E2–25K (35).

**Rabbit UbcH5A**—Plasmid pET3a-UbcH5A (34) was provided by P. Howley (Harvard Medical School). UbcH5A was expressed in E. coli strain BL21(DE3)pLysS at 30 °C as described previously (35). Cell pellets were frozen overnight at −20 °C, then thawed and resuspended in lysis buffer (33) using 2 ml of buffer/g of cells. Lysis and DNA digestion were carried out as described (33). After centrifugation at 10,000 × g for 20 min, the supernatant was fractionated with ammonium sulfate. Proteins precipitating between 50 and 80% saturation were collected and dialyzed against buffer containing 25 mM Tris-HCl (5% base), 0.1 mM EDTA, and 0.5 mM DTT. The dialysate was passed through an S-Sepharose column (20 mg protein/ml resin) that had been equilibrated with buffer containing 50 mM Tris (5% base), 0.1 mM EDTA, and 0.5 mM DTT. The column was washed with 2 volumes of the same buffer and then eluted with 4 volumes of the same buffer containing 50 mM NaCl. The 50 mM NaCl eluate was concentrated by ultrafiltration (Millipore Ultrafree-4) and chromatographed on a 1 × 46 cm Sephacryl-200 column equilibrated with S buffer. Fractions of 1 ml were collected, and thiol ester assays were used to locate the peak of UbcH5A.

**Other E2s**—E2–14K, E2–20K, and E2–35K were purified from rabbit reticulocyte lysate (27). Recombinant C170S-E2–25K (33) and HsUbc13 (22) were purified as described.

**Ub5 Synthesis Assay**—Ub5 synthesis was assayed by combining purified E1 with an E2 source (fraction I, crude rabbit Ubc5, or purified recombinant UbcH5A) and an E3 source (see figure legends). Unless otherwise indicated, assays contained 0.5 μM E2 and 0.15 mg/ml E3 protein. Assays were initiated by adding a mix contributing 50 mM Tris-HCl (24% base), pH 7.6, 5 mM MgCl2, 2 mM ATP (and a regenerating system), 0.3 units/ml inorganic pyrophosphatase, ~0.5 mM DTT, ~0.1 μM E1, 1 mM MgCl2 and 2 μM Ub (usually K48R). In some cases UbA (1 μM) was added to inhibit isopeptidases (see figure legends). Reactions were incubated for times ranging from several min to 90 min (37 °C), quenched with sample buffer, and resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography and quantified by band excision and γ-counting (by phosphoimage analysis). The UbcH5A concentration dependence of E3 activity was investigated only in a preliminary way. The dependence was apparent hyperbolic, with a K50 of ~50–100 nM.

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2 J. Piotrowski and C. Pickart, unpublished data.

3 J. You, R. E. Cohen, and C. M. Pickart (1999) *BioTechniques*, in press.

4 J. You and C. Pickart, unpublished data.
interaction of E2–14K and E3s follows a $K_m$ of 70 to 200 nM (36, 37). Kinetic analysis of Ub$_2$ synthesis was performed by varying the concentration of K48R-Ub$_{74}$ in incubations of 60-min duration. Data were analyzed by plotting $V_{\text{max}}$ versus [K48R-Ub$_{74}$]. In addition, $\log(V_{\text{max}})/V_{\text{max}}$ was plotted against $\log(K48R-Ub_{74})$ to obtain the Hill parameters $n_H$ and $K_H$

Chain Synthesis Using “Single-lysine” Ub—Incubations with E2–25K (see Fig. 5A, “Results”) were carried out for 30 min at 2 mg/ml Ub and 20 $\mu$m C170S-E2–E2–25K (pH 8, 37 °C) as described (33). Incubations to assess linkage specificity in Ub$_2$ synthesis by the E2/E3 were carried out at pH 7.3 and 37 °C under conditions described in the legend to Fig. 5B (see “Results”). Incubations to assess linkage specificity in polyUb chain synthesis by the E2/E3 were carried out at pH 7.3 and 37 °C as described in the legend to Fig. 5C (see “Results”). In the first two cases, product formation was detected by SDS-polyacrylamide gel electrophoresis and Coomassie staining. In the third case, a reaction aliquot was analyzed by Western analysis (ECL detection) with affinity-purified anti-Ub antibodies (38).

RESULTS AND DISCUSSION

Enzymes in Fractions I and II Cooperate to Assemble PolyUb Chains Linked Through a Novel Lysine Residue(s)—Bamezai and Breslow (25) report the synthesis of unanchored polyUb chains in a manner dependent on a factor(s) in fraction I (neutral/cationic proteins) and a factor(s) in fraction II (anionic proteins) of rabbit reticulocyte lysate. The requirement for fraction I indicated that these were not K48-linked chains assembled by the well characterized enzyme E2–25K, because E1 and E2–25K are both in fraction II (39).

To address whether unanchored chains synthesized under these conditions (i.e., in fraction I plus fraction II) were linked through a lysine residue other than Lys-48, we used a polyUb chain assembly assay involving a low concentration of $^{125}$I-K48R-Ub and a high concentration of K48R-Ub$_{74}$. The assay also contained purified E1. In this assay, competition effects will strongly favor the use of K48R-Ub$_{74}$ (versus K48R-Ub) as the initiating Ub and counteract the formation of Ub$_2$ and higher chains (since K48R-Ub$_{74}$ lacks the C-terminal Gly-Gly dipeptide, it can only serve as the proximal Ub in the chain, i.e., as the donor of a lysine residue). Thus, Ub$_2$ is formed in a manner that is linear versus time (e.g., Fig. 4, below). Moreover, since both Ub derivatives lack lysine at residue 48, the assay detects only novel dimers. Having Ub$_{74}$ as the proximal Ub may also help to stabilize the Ub$_2$ product against disassembly by isopeptidase T (40), although we did not directly test the susceptibility of the novel chains to disassembly by deubiquitinating enzymes.

Using this assay, we found that the synthesis of a 17-kDa $^{125}$I-labeled product (presumptive Ub$_2$) was strongly stimulated when we combined fraction I and fraction II (data not shown). Since fraction I is known to contain a number of E2s, including members of the Ubc5 subfamily (34, 41), we next determined whether fraction I could be replaced by a mixture of E2s purified from fraction I by Ub affinity chromatography. The E2 preparation contained a prominent 16-kDa protein(s) that formed a thiol ester with Ub (see “Experimental Procedures”). One or more Ubc5 homolog(s) was present, based on the observation of a strong 16-kDa band in immunoblots developed with antibodies against Drosophila Ubc5 (data not shown). We will refer to this material as a crude Ubc5 preparation. Reconstitution assays revealed that this preparation contained an enzyme(s) that supported Ub$_2$ synthesis in the absence of added fraction II (Fig. 1, lane 2). However, greater than additive synthesis of Ub$_2$ was seen when the Ub$_2$ preparation was combined with fraction II (Fig. 1, lane 3 versus lanes 1 and 2). Because both Ub molecules in the assay carried the K48R mutation, this Ub$_2$ product was atypically linked. The formation of high molecular weight conjugates was also strongly stimulated when the crude Ubc5 preparation was combined with fraction II (Fig. 1, lane 3). Presumably these large products reflect the conjugation of $^{125}$I-K48R-Ub to endogenous proteins in fraction II, catalyzed by endogenous E3s. Ubc5 isoforms are known to function with several different E3s (17, 42–44).

These reconstitution studies showed that fraction I and fraction II each contained an enzyme necessary for the synthesis of a novel Ub$_2$ product. The covalent affinity procedure used to generate the E2 preparation yielded an apparent rabbit Ubc5 homolog(s) (above). A simple interpretation of the reconstitution results is that Ub$_2$ synthesis depended on a Ubc5 E2 contributed by fraction I and on an E3 contributed by fraction II. The experiments described below strongly support this interpretation.

Biochemical Characterization of E3 Activity—To further characterize the presumptive E3, fraction II was subjected to stepwise anion exchange fractionation as described under “Experimental Procedures.” Fig. 2A shows that the putative chain-synthesizing E3 bound to the column (lane 1 versus lane 2) and eluted predominantly in the 0.4 M NaCl fraction (lane 6). This activity depended on the addition of crude Ub$_5$ (lane 6 versus lane 9). Based on its strong binding to the MonoQ column at neutral pH, the E3 is an acidic protein. Proteins in the peak fraction from the anion exchange column were next resolved on a gel filtration column. Ub$_2$ synthesis activity eluted in a peak centered at $\pm 120$ kDa (filled circles, Fig. 2B; these assays employed recombinant UbcH5A, see legend). Ub$_2$ synthesis was strictly dependent upon added Ub$_5$ (filled versus open circles, Fig. 2B). The pooled peak fractions were concentrated and used as the source of E3 in most of the experiments described below. The molecular masses of known E3s range from 100 to 200 kDa (3). The size of the partially purified enzyme is thus consistent with it being an E3.

Certain E3s bearing a conserved “Hect” domain at their C termini form thiol ester adducts with Ub in the presence of E1 and their cognate E2s (43, 45). We did not detect a labile Ub adduct of a high molecular mass ($\approx 100–200$ kDa) when the E3 was assayed for thiol ester formation in the presence of E1 and Ub$_5$ (data not shown). However, the E3 may catalyze chain synthesis through a thiol ester intermediate whose level was too low to detect. Coomassie Blue staining of an SDS-polyacrylamide gel electrophoresis gel revealed multiple high molecular weight bands in the peak fractions from the gel filtration column (data not shown), suggesting that the E3 polypeptide(s) constitutes only a low fraction of the total protein.

**Fig. 1. Synthesis of Ub$_2$ linked through novel lysine residue (autoradiograph).** Ub$_2$ synthesis was assayed in incubations of 60-min duration. Besides core assay components (see “Experimental Procedures”), the assays contained fraction II protein at 2 mg/ml and/or crude rabbit Ub$_5$ at 2 $\mu$m. Lane 1, fraction (Fr.) II alone; lane 2, crude Ub$_5$ alone; lane 3, fraction II plus crude Ub$_5$.
specific interaction between the E3 and UbcH5A, a family can indeed cooperate with the E3. Consistent with a recombinant E2 indicates that an enzyme of the Ubc5 panel B concentrations; the crude E2 was used in panel A. In the experiments described above the crude Ubc5 preparation gave a low but detectable rate of product formation in the absence of E3 (Fig. 1, lane 2). To minimize this E3-independent reaction, we expressed the human Ubc5 homolog known as UbcH5A (34, 46) in E. coli as described under “Experimental Procedures”. All-ubiquitin chains under physiological conditions, we varied the concentration of K48R-Ub74 and measured the rate of Ub2 synthesis. We first showed that Ub2 formation at 1 mg/ml K48R-Ub74 depended linearly on E3 concentration (at 0.3 μM UbcH5A; Fig. 3A) and time (up to 90 min at 0.3 μM UbcH5A and 0.15 mg/ml E3 protein, not shown). The substrate concentration dependence was then determined at concentrations of K48R-Ub74 ranging from 0.7 μM to 60 μM. Saturation was evident above 25 μM, whereas the data at lower acceptor concentrations were best fit by a sigmoid (versus hyperbolic) dependence. The line in Fig. 3B assumes \( K_{m,app} = 8.7 \mu M \) and \( n_H = 1.8 \). The total concentration of Ub in cells is typically ~20 μM, with about 50% in the unconjugated form (38). The value of \( K_{m,app} \) is below the physiological concentration of free Ub, predicting a significant rate of Ub2 synthesis under conditions pertaining in cells.

**Fig. 2. Biochemical characterization of chain-synthesizing E3.** A, stepwise anion exchange (autoradiograph). Fraction II protein (10 mg) was fractionated on a fast protein liquid chromatography MonoQ column (see “Experimental Procedures”). Volume-normalized aliquots (0.2%) of the indicated fractions were assayed for Ub2 synthesis in incubations of 60-min duration (see “Experimental Procedures”). Lane 1, fraction II (load (L)); lane 2, flow-through (FT); lanes 3–10, salt fractions as indicated. Lanes 1–7, plus crude Ubc5 (0.3 μM); lanes 8–10, no added Ubc5. The positions of Ub1 and Ub2 are indicated. The Ub2 seen in lane 4 is probably Lys-63-linked, reflecting the elution of rabbit Ubc13 and associated Ub E2 variant proteins in this fraction (22). B, gel filtration. The 0.4 M NaCl fraction (0.19 ml) from a MonoQ column (as in panel A, except that 14 mg of fraction II protein was loaded) was fractionated on Sephacryl-200 (see “Experimental Procedures”). All-ubiquitin fractions as indicated.

Recombinant UbcH5A Reconstitutes Novel Ub2 Synthesis—In the experiments described above the crude Ubc5 preparation gave a low but detectable rate of product formation in the absence of E3 (Fig. 1, lane 2). To minimize this E3-independent reaction, we expressed the human Ubc5 homolog known as UbcH5A (34, 46) in E. coli as described under “Experimental Procedures.” Volume-normalized aliquots (0.2%) of the indicated fractions were assayed for Ub2 synthesis in incubations of 60-min duration (see “Experimental Procedures”). Lane 1, fraction II (load (L)); lane 2, flow-through (FT); lanes 3–10, salt fractions as indicated. Lanes 1–7, plus crude Ubc5 (0.3 μM); lanes 8–10, no added Ubc5. The positions of Ub1 and Ub2 are indicated. The Ub2 seen in lane 4 is probably Lys-63-linked, reflecting the elution of rabbit Ubc13 and associated Ub E2 variant proteins in this fraction (22). B, gel filtration. The 0.4 M NaCl fraction (0.19 ml) from a MonoQ column (as in panel A, except that 14 mg of fraction II protein was loaded) was fractionated on Sephacryl-200 (see “Experimental Procedures”). All-ubiquitin fractions as indicated.

**Fig. 3. Kinetic studies.** A, dependence of rate on E3 concentration. Assays of 30-min duration were carried out using 0.3 μM purified recombinant UbcH5A and the indicated concentration of E3 protein (8200 peak). Data were corrected using a blank obtained by omitting K48R-Ub74 from the assay. A similar linear dependence was seen in 90-min assays. B, dependence of rate of Ub2 synthesis on [K48R-Ub74]. Assays of 60-min duration contained purified recombinant UbcH5A (0.3 μM), partially purified E3 (0.15 mg/ml, 5200 peak), and [125I]-K48R-Ub (3 μM). Data were corrected using a blank obtained by omitting K48R-Ub74 from the assay. Data from four experiments are combined; in each experiment, rates were normalized to the rate seen at 25 μM acceptor (\( V_{max} \)) in the same experiment. K48R-Ub74 was added at the concentration indicated on the abscissa. The open circles and line represent a fit to the data obtained using the Hill equation assuming \( K_{m,app} = 8.7 \mu M \) and \( n_H = 1.8 \) (see “Experimental Procedures”). UbcH5A in assays of Ub2 synthesis (see “Experimental Procedures”). In addition, we tested five other purified E2s for the ability to reconstitute E3-dependent Ub2 synthesis (E2–14K, E2–20K, E2–25K, E2–35K, and Ubc13); all were found to be inactive (data not shown).

**Kinetics of Ub2 Synthesis**—The assays described above were carried out at a high concentration of acceptor Ub (1 mg/ml = 117 μM). To address whether these enzymes could assemble chains under physiological conditions, we varied the concentration of K48R-Ub74 and measured the rate of Ub2 synthesis. We first showed that Ub2 formation at 1 mg/ml K48R-Ub74 depended linearly on E3 concentration (at 0.3 μM UbcH5A; Fig. 3A) and time (up to 90 min at 0.3 μM UbcH5A and 0.15 mg/ml E3 protein, not shown). The substrate concentration dependence was then determined at concentrations of K48R-Ub74 ranging from 0.7 μM to 60 μM. Saturation was evident above 25 μM, whereas the data at lower acceptor concentrations were best fit by a sigmoid (versus hyperbolic) dependence. The line in Fig. 3B assumes \( K_{m,app} = 8.7 \mu M \) and \( n_H = 1.8 \). The total concentration of Ub in cells is typically ~20 μM, with about 50% in the unconjugated form (38). The value of \( K_{m,app} \) is below the physiological concentration of free Ub, predicting a significant rate of Ub2 synthesis under conditions pertaining in cells.
The value of $V_{\text{max}}$ calculated from Fig. 3B, $54 \pm 5$ pmol Ub/min/mg of E3 protein, cannot yet be interpreted because we do not know the concentration of the E3. If the E3 is 10% of the total protein and assuming a molecular mass of 100 kDa, the value of $k_{\text{cat}}$ would be $0.06 \text{ min}^{-1}$. If the E3 is less abundant, as we think likely, $k_{\text{cat}}$ would be larger. Reported $k_{\text{cat}}$ values for E2s in E3-independent conjugation range from 0.02 to 0.6 min$^{-1}$ (27, 33, 47), whereas $k_{\text{cat}}=-0.5 \text{ min}^{-1}$ has been reported for the E2-E3 hybrid protein E2–230K (48). No $k_{\text{cat}}$ value has been reported for an E3.

**Influence of Residue 48 on Utilization of Novel Lysine Residue**—To address which lysine residue of Ub was utilized in Ub$_2$ synthesis, we tested them as substrates for chain assembly by E2–25K, an enzyme that exhibits strong specificity for Lys-48 (39). As shown in Fig. 5A, the kinetics of chain synthesis were qualitatively similar for wild-type and 48K-Ub, whereas Ko-Ub and all of the other derivatives were essentially inactive as substrates. Some differences in the electrophoretic migration of Ub$_2$ and higher species were evident for wild-type versus 48K-Ub (lane 2 versus lane 6), possibly reflecting conformational differences due to the presence of $S$-aminohexlycysteine at the linkage site, $N,N$-dimethyllysine at other sites, differences in the levels of cyclized chains, or a combination of these effects. The strong activity of E2–25K toward 48K-Ub and its negligible activity toward the other Ub$_2$ (Fig. 5A) confirm that the $N$-methylolation and alkylation of each Ub proceeded essentially to completion.

The same single-lysine derivatives were used in chain assembly assays with the E2 and E3 described above. For these experiments, we used higher enzyme concentrations than in most other assays (legend, Fig. 5B) and detected the products by Coomassie staining. A control with wild-type Ub showed that chains containing up to five Ubs were detectable after 4 h of incubation (Fig. 5B, lane 1 versus lane 2). We expected that (at least) two lysines would be used as linkage sites (above), and this proved to be the case. As shown in Fig. 5B, substantial and qualitatively similar levels of Ub$_2$ were produced from 29K-Ub (lane 10) and 48K-Ub (lane 12). Controls showed that no Ub$_2$ was synthesized from wild type, 29K-, or 48K-Ub if the E3 was omitted from the incubation (data not shown). These results indicate a high likelihood that Lys-29 is the predominant site of conjugation in K48R-Ub, as well as the site whose utilization is inhibited by the presence of cysteine at residue 48 (above). However, the identification of Lys-48 as a conjugation site was unexpected, since the E3 was purified based on its ability to ubiquitinate a lysine residue other than Lys-48.

To confirm that the K48C mutation was inhibitory for chain synthesis, we treated K48C-Ub with trypsin (49) to make K48C-Ub$_{74}$. This derivative was used as the acceptor in Ub$_2$ synthesis assays with K48R-Ub. As seen in Fig. 4 (filled versus open circles), the presence of cysteine (versus arginine) at residue 48 reduced the initial rate of Ub$_2$ synthesis by $\approx 5$-fold. The inhibitory effect of the K48C mutation was not due to the presence of cysteine per se, because identical rates of Ub$_2$ synthesis were seen when K29C-Ub$_{74}$ and K48R-Ub$_{74}$ were compared as acceptors (at 1 mg/ml, data not shown). These results indicate that the presence of cysteine (versus arginine) at residue 48 specifically inhibited the ubiquitination of a lysine residue other than Lys-48. Further studies will be needed to determine whether the K48C mutation affects substrate binding versus the ubiquitination of the bound substrate. This behavior indicates a need for caution when interpreting the effects of lysine point mutations in Ub. Inhibitory effects in such experiments are usually taken to mean that the mutated lysine is a site of Ub-Ub conjugation. In the present case, the ubiquitination that was inhibited occurred at a different lysine residue from the one that was mutated.

**Lys-29 and Lys-48 Are Utilized in Chain Assembly**—In reactions with K48C-Ub as acceptor, the observed inhibition was due to an effect on the utilization of a non-Lys-48 lysine residue. However, this second lysine was not the sole site of ubiquitination, because in this case, placing a Cys at this second lysine as well as at residue 48 should have blocked Ub$_2$ synthesis. Instead, K6C, K11C, K29C, K48R, and K63C-Ub were all well utilized in chain assembly (above). Taken together, these results suggested that there were at least two sites of Ub-Ub conjugation. To identify the relevant lysines, we used chemical modification to prepare Ub derivatives harboring only one conjugation site each. Specifically, we reductively $N$-methylated each of the five Lys-to-Cys mutants (above) to block every lysine residue (and the N-termini) and then alkylated the Cys residue in each protein with ethyleneimine to introduce a lysine mimic, $S$-aminohexlycysteine (4, 5, 10). In the case of K48C-Ub, a polyUb chain assembled from the final product of these manipulations is a competent degradation signal (5).

For convenience, these single-lysine derivatives are named based on the position of the lysine mimic, i.e. 48K-Ub is the product derived from K48C-Ub. Ko-Ub denotes the product derived from wild-type Ub; Ko-Ub should be devoid of lysine residues. To assess the integrity and quality of these derivatives, we tested them as substrates for chain assembly by E2–25K, an enzyme that exhibits strong specificity for Lys-48 (39). As shown in Fig. 5A, the kinetics of chain synthesis were qualitatively similar for wild-type and 48K-Ub, whereas Ko-Ub and all of the other derivatives were essentially inactive as substrates. Some differences in the electrophoretic migration of Ub$_2$ and higher species were evident for wild-type versus 48K-Ub (lane 2 versus lane 6), possibly reflecting conformational differences due to the presence of $S$-aminohexlycysteine at the linkage site, $N,N$-dimethyllysine at other sites, differences in the levels of cyclized chains, or a combination of these effects. The strong activity of E2–25K toward 48K-Ub and its negligible activity toward the other Ubs (Fig. 5A) confirm that the $N$-methylation and alkylation of each Ub proceeded essentially to completion.

In Fig. 5B, chains of $n > 2$ were not strongly detected except in the reaction with wild-type Ub (compare lanes 2, 10, and 12). To increase the sensitivity of chain detection, we carried out incubations at lower concentrations of enzymes and Ub.
monitored product formation by Western blot analysis with anti-Ub antibodies (38). For these experiments, we employed recombinant Ub molecules carrying one lysine residue each (other lysines mutated to arginines; see “Experimental Procedures”). For simplicity, these mutant proteins are named based on the site of the lysine residue (e.g. Lys-29-Ub has a lysine at residue 29). As shown in Fig. 5C, Lys-29-Ub and Lys-48-Ub were each assembled into long chains in incubations containing 20 \( \mu \text{M} \) Ub, a physiological concentration. There was also very weak activity toward Lys-63-Ub. This experiment was conducted in the absence of Ubal to avoid a high background of chain assembly due to the use of Ubal as an acceptor. Differences in rates of chain disassembly are thus likely to contribute to the higher yield of products seen with Lys-29-Ub versus Lys-48-Ub in Fig. 5C.

K29C- and K48R-Ub\(_{74}\) (1 mg/ml) were conjugated at identical rates (above), and we observed a qualitatively similar concentration dependence in assays of Ub\(_{2}\) synthesis with each acceptor. The latter results provide a preliminary indication that \( V_{\text{max}} \) and \( K_m \) values are similar for the ubiquitination of Lys-48 and Lys-29. Thus, both sites should be ubiquitinated in vivo. We have shown that the enzymes can assemble chains through either Lys-29 or Lys-48 (Fig. 5C). It will be shown below that they can also assemble chains harboring both linkages.

**Chain Elongation**—The assembly of long unanchored chains from mono-Ub (Fig. 5C) requires that the enzymes can transfer mono-Ub to a growing chain or transfer a chain to mono-Ub or both. To test whether a chain is a functional acceptor, we used the two Lys-48-linked Ub\(_2\) molecules shown schematically in Fig. 6A. Each chain had a Asp-77 cap at its proximal terminus (10), permitting it to be substituted for Ub\(_2\) in the assay described above. Chain b had a cysteine residue at position 48 in the distal Ub, whereas chain c had S-aminoethylcysteine at this position. As shown in Fig. 6B, lanes 2 and 3, both tetra-
mers were substrates for ubiquitination. Moreover, the rate of product formation was comparable with that seen at the same concentration (20 μM) of K48R-Ub74 (compare with lane 1). The results of qualitative concentration dependence studies suggest that $K_m$ and $V_{\text{max}}$ values for chains b and c are fairly similar to those for K48R-Ub74. Thus, chains are not favored as acceptors. The results shown in Fig. 6B also bear on chain topology. Because chain b is devoid of Lys-48 residues (Fig. 6A), the finding that it is a good acceptor indicates that the enzymes can efficiently ubiquitinate Lys-29 in a Lys-48-linked chain, i.e., there is no evident block to the assembly of chains containing both Lys-29 and Lys-48 linkages. It is not known which of the four Lys-29 residues in chain b is the ubiquitination site. Although the utilization of the distal Lys-29 should be inhibited due to the presence of C48 (Fig. 4), the other three Lys-29 residues may be even more deficient as acceptors due to the involvement of the corresponding Lys-48 residues in isopeptide bonds. The similar concentration dependence shown by the two chains versus K48R-Ub74 suggests that only one of the Ub moieties in each chain is recognized by the E3. Based upon these considerations, we think it likely that the distal Ub is ubiquitinated in each of these chains. However, further studies will be needed to confirm this interpretation.

The ability of the enzymes to use a chain as an acceptor suggests that they can lengthen a chain conjugated to a substrate protein. As a further test of this possibility, we determined whether the enzymes could ubiquitinate a linear Ub-DHFR fusion protein. Ub-DHFR (20 μM) was indeed a substrate (lane 7 of Fig. 6B). Although the level of the mono-ubiquitinated product was somewhat lower than the level of the corresponding product in assays with K48R-Ub74 and chain c (compare lane 7 to lanes 5 and 6), the low yield of Ub74-DHFR was in part due to its conversion to more highly ubiquitinated forms, which are faintly visible as a ladder above Ub74-DHFR.

Concluding Remarks—The principal finding of this study is that free Ub can be efficiently recognized as a substrate for polyUb chain assembly by enzymes in a mammalian cell extract. Chain assembly could be reconstituted with three enzymes: an apparent E3, the E2 UbcH5A, and the E1 enzyme that is required in all ubiquitination reactions. Chain synthesis by these enzymes follows $K_m$ ~9 μM for the acceptor Ub (modeled by Ub74 in Fig. 3B and by Ub in Fig. 6B). The $K_m$ for the Ub undergoing transfer, although not determined, is probably much lower, reflecting the low $K_m$ of Ub in the E1 reaction (<1 μM (50)), the high efficiency of Ub transfer from E1 to E2s (47, 51), and the efficient interactions of E2s with their cognate E3s (e.g. 36, 37, and 44). Its kinetic parameters argue that Ub is a physiological substrate of these enzymes and, thus, that chain assembly is a physiological activity. However, it remains possible that the enzymes have substrates besides Ub; for example, stable Ub fusion proteins, if present at a concentration high enough to compete with Ub/polyUb chains, should be substrates (Fig. 6B). Besides mono-Ub and unanchored polyUb chains, a Ub molecule already conjugated to a substrate lysine residue can serve as substrate for chain elongation, as suggested by the acceptor competence of Ub74 and Ub-DHFR (Fig. 6B). Taken together, our results suggest that the enzymes described here may act to facilitate substrate polyubiquitination in a general manner through either of the two mechanisms outlined in Fig. 6C. Because mono-Ub and “conjugated” Ub (consisting of unanchored polyUb chains and substrate-linked Ub/polyUb) are present at ~10 μM each in mammalian cells (38), both of the assembly pathways in Fig. 6C are predicted to operate in vivo.

What biological purpose could be served by Ub-specific conjugating enzymes? When the products are Lys-48-linked chains, their actions may serve to finalize the decisions made by substrate-specific Ub-conjugating enzymes. Because Lys-48-linked chains of $n < 4$ have a low affinity for the proteasome (10), a substrate is likely to escape degradation if its cognate E2-E3 enzymes fail to ligate at least four Ub's. The actions of the enzymes described here could avoid such an outcome. Whether Ub-specific enzymes pre-assemble chains for use by substrate-specific enzymes (Fig. 6C, left) or lengthen short chains previously assembled on target proteins by substrate-specific enzymes (Fig. 6C, right), the activity of the Ub-specific enzymes will tend to facilitate the degradation of the target proteins selected by the substrate-specific enzymes. In view of the low level of unanchored polyUb chains in mammalian cells (13), the high susceptibility of K48-linked chains to disassembly by isopeptidase T (40), and the ability of Lys-48-linked chains to inhibit the proteasome through competition effects (10, 16), we speculate that sequential assembly may be favored in vivo. A sequential mechanism of chain assembly could also afford additional opportunities for maximizing specificity in ubiquitination, for example through interactions between substrate-specific and ubiquitin-specific E2-E3 complexes. Separating substrate recognition and Ub recognition could also provide a mechanistic advantage by avoiding a requirement that the substrate-specific E3 bind two distinct acceptors, the substrate and Ub, with high affinity. However, the proposed separation of the substrate and ubiquitin ligation steps is unlikely to apply in all cases, because certain substrate-specific E3s, such as E3α/Ubr1, can polyubiquitinate target proteins in an autonomous and processive manner (52).

The properties of the chain-assembling E2-E3 contrast with the properties of the only other chain-assembling enzyme that has been kinetically characterized, namely E2–25K. The $K_m$ of the acceptor Ub in the E2–25K reaction, ~600 μM (33), is more than 50-fold larger than the $K_m$ of the conjugation system described here. At 10 μM Ub, a physiological concentration, the E2–25K reaction will proceed at ~2% of its maximum rate, whereas the above-described enzymes will operate at about half their maximum rate. These considerations reinforce a prior conclusion that unassisted chain assembly is not the biological activity of E2–25K (33).

The partially purified E3 assembles chains through two different lysine residues (Lys-29 and Lys-48). Although this finding is most simply explained if the preparation harbors two E3 enzymes with distinct linkage specificities, we favor the interpretation that there is a single E3 with a dual linkage specificity for a number of reasons. First, the two activities co-migrate through several chromatographic steps (see “Experimental Procedures”; Fig. 5C). Second, the ubiquitinations of Lys-48 and Lys-29 occur with very similar kinetics. Third, the ubiquitination of Lys-29 is sensitive to the identity of residue 48, suggesting that the conjugation to Lys-29 requires interaction with Lys-48 (Fig. 4). Fourth, if different E3s acted on Lys-48 versus Lys-29, then the $V_{\text{max}}$ of wild-type Ub should be larger than that of K48R- or K29C-Ub, because wild-type Ub would be utilized by both enzymes, whereas each mutant Ub would be utilized by only one enzyme. Instead, wild-type, K48R-, and K29C-Ub are ubiquitinated with identical $V_{\text{max}}$ values (Fig. 4, filled circles versus squares, and data not shown). Finally, the properties of yeast Ufd4p provide precedent for a dual linkage specificity: in yeast cells, Ufd4p ubiquitinates either Lys-29 or Lys-48 of a linear Ub-Pro-β-galactosidase fusion protein (17). However, a rigorous determination of whether the mammalian E3 preparation harbors one or two enzymes awaits purification of the relevant enzyme(s) to homogeneity.

Jentsch and co-workers (18) show that purified yeast Ufd4p
cooperates in vitro with Ubc4p to assemble short polyUb chains on Ub fusion proteins, with the first Ub linked to Lys-29 of the fused Ub. These investigators proposed that the Ufd2p-mediated assembly of long chains, which requires Ufd2p, involves Lys-48. The similarity in E2 and linkage specificities raises the possibility that the enzyme(s) described here could represent a Ufd2p-like E3(s), perhaps acting in conjunction with a mammalian Ufd2p homolog. Further purification of the mammalian enzyme will be necessary to determine whether this is the case.

These results provide the first direct demonstration of Lys-29-linked polyUb homopolymers (Fig. 5C) and the first direct demonstration of polyUb heteropolymers (Fig. 6B). The kinetic data suggest that the ubiquitination of Ub residue Lys-29 should occur with significant frequency and raise the question of whether homo- and heteropolymers harboring Lys-29 linkages are competent signals for proteasomal degradation. Vegetatively growing yeast cells expressing the K29R mutant as ages are competent signals for proteasomal degradation. Veg-

References

Acknowledgments—We thank R. Hofmann for conducting preliminary studies and for providing purified Ub4, proteins and E. Kasperek for help in purifying rabbit Ubc5. For generously providing reagents, we nary studies and for providing purified Ub 74 proteins and E. Kasperek 27306

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E3-mediated Assembly of Lys-29-linked PolyUb Chains

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