Novel Multiubiquitin Chain Linkages Catalyzed by the Conjugating Enzymes E2<sub>EPF</sub> and RAD6 Are Recognized by 26 S Proteasome Subunit 5*

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Targeting of substrates for degradation by the ATP, ubiquitin-dependent pathway requires formation of multiubiquitin chains in which the 8.6-kDa polypeptide is linked by isopeptide bonds between carboxyl termini and Lys-48 residues of successive monomers. Binding of Lys-48-linked chains by subunit 5 of the 26 S proteasome regulatory complex commits the attached target protein to degradation with concomitant release of free ubiquitin monomers following disassembly of the chains. Point mutants of ubiquitin (Lys → Arg) were used to map the linkage specificity for ubiquitin-conjugating enzymes previously demonstrated to form novel multiubiquitin chains not attached through Lys-48. Recombinant human E2<sub>EPF</sub> catalyzed multiubiquitin chain formation exclusively through Lys-11 of ubiquitin while recombinant yeast RAD6 formed chains linked only through Lys-6. Multiubiquitin chains linked through Lys-6, Lys-11, or Lys-48 each bound to subunit 5 of partially purified human 26 S proteasome with comparable affinities. Since chains bearing different linkages are expected to pack into distinct structures, competition between Lys-11 and Lys-48 chains for binding to subunit 5 demonstrates that the latter possesses determinants for recognizing alternatively linked chains and precludes the existence of subunit 5 isoforms recognizing distinct structures. In addition, competition studies provided an estimate of K<sub>D</sub> ~ 18 nM for the intrinsic binding of Lys-48-linked chains of linkage number n > 4. This result suggests that the principal mechanistic advantage of multiubiquitin chain formation is to enhance the affinity of the associated substrate for the 26 S complex relative to that of unconjugated target protein. Complementation studies with E1/E2-depleted rabbit reticulocyte extract demonstrated RAD6 supported isopeptide ligase-dependent degradation only through Lys-48-linked chains, while E2<sub>EPF</sub> retained the ability to target a model radiolabeled substrate through Lys-11-linked chains. Therefore, the linkage specificity exhibited by these E2 isozymes depends on their catalytic context with respect to isopeptide ligase.

ATP-dependent conjugation of ubiquitin to protein targets is currently recognized to mediate a variety of cellular processes by signaling selective degradation of the latter through the 26 S proteasome pathway, reviewed most recently in Refs. 1 and 2. Among the cellular targets serving as substrates for this unique post-translational modification are various proteins exhibiting either constitutive or conditional short half-lives including cyclins (3–8), various oncoproteins (9–12), p53 (13–15), transcriptional factors (16–18), and proteins of abnormal structure (19–21). In all cases, the signal for ubiquitination probably requires transient exposure of one or more lysines that can serve as sites for recognition and attachment of the polypeptide. For certain targets, enhanced steric accessibility of sensitive lysines arising by minute conformational changes (22–24) or more global folding transitions (21, 25) may be accompanied by unmasking of specific amino-terminal residues that dispose the protein to recognition by relevant isopeptide ligases (E3) that confer specificity (4, 26–30). In the case of cyclins, discrete recognition signals are conserved among related isoforms and within unrelated proteins (6, 31), although the precise mechanism by which these sequences contribute to substrate recognition by relevant conjugating enzymes has not been well elucidated.

Attachment of single ubiquitin moieties to target proteins effects a modest rate of degradation by the 26 S proteasome (32–34); however, more robust signals for degradative targeting require subsequent formation of multiubiquitin homopolymers by chain elongation from the initial polypeptide conjugate (32, 33, 35). Considerable recent work has demonstrated that these multiubiquitin chains are formed by a repeating structure in which the carboxyl terminus of each ubiquitin is linked to Lys-48 of the preceding ubiquitin (33, 35). The crystal structure of the resulting multiubiquitin chain exhibits considerable packing order and symmetry that is thought essential for recognition by the 55 subunit of the regulatory complex capping the 26 S proteasome (36, 37). This model is supported by mutagenesis studies identifying essential ubiquitin residues required for both multiubiquitin chain binding to 55 and for subsequent degradative targeting (38).

Ubiquitin-mediated proteolysis has been most extensively studied in yeast and rabbit reticulocytes. Within these systems, the almost quantitative inhibition of ATP-dependent degradation accompanying substitution of rmUb<sub>4</sub> or UbK<sub>48R</sub> for wild type polypeptide demonstrates that a significant fraction of degradative flux proceeds through conjugated intermediates bearing Lys-48-linked multiubiquitin chains since neither rmUb nor UbK<sub>48R</sub> supports chain elongation (33, 35). However, mounting evidence supports the existence of mult ubiqui-
utin chains bearing linkage specificities distinct from Lys-48. Purified recombinant yeast RAD6, a member of the ubiquitin carrier protein (E2) isozyme family, catalyzes multiquitin chain formation to core histones in the presence of only ubiquitin activating enzyme (E1) to maintain the E2 active site cysteine charged with ubiquitin thioester (39). The linkage specificity for these chains does not require Lys-48 since rmUb but not UbK48R blocks the characteristic ladder of conjugates revealed by SDS-PAGE (39). Similar results supporting Lys-48-independent chains have more recently been obtained with recombinant E2$_{EPF}$, an isoform cloned from human keratinocytes using autoantibodies obtained from pemphigous foliaceus patients (64). Finally, stable Lys-63-linked chains requiring participation of RAD6 (UBC2) have been observed in yeast and proposed to account in part for the DNA repair function of this E2 isoform (40). Since RAD6 normally supports Lys-48 chain-dependent degradation in yeast within the N-end rule pathway (41, 42), the latter observation suggests linkage specificity may be context specific and depend on either the target protein or, more likely, the cognate E3 required for conjugation.

The currently accepted paradigm for target protein conjugation requires ubiquitin activating enzyme, a ubiquitin carrier protein, and ubiquitin:protein isopeptide ligase. The metabolic significance of E3-independent conjugation by certain members of the E2 family remains uncertain, although conjugates formed in the absence of E3 by yeast RAD6 and CDC34 as well as the rabbit reticulocyte isoform E2$_{32K}$ are substrates for 26 S proteasome-mediated degradation (32). In the present studies, mutagenesis of the lysine residues present on ubiquitin have allowed the assignment of linkage specificity for multiquitin chain formation by RAD6 and E2$_{EPF}$. Other results indicate that these alternatively linked chains are recognized by subunit 5 of the 26 S proteasome, suggesting target proteins marked by such homopolymer structures may be degradative intermediates. Finally, reconstitution experiments with rabbit reticulocyte extracts demonstrate that alternatively linked chains are competent in the overall degradative pathway. These results define a functional role for alternatively linked chains and serve as a basis for future mechanistic studies with the cognate E3 isoforms.

**MATERIALS AND METHODS**

Inorganic pyrophosphatase (high pressure liquid chromatography purified) was obtained from Sigma. Carrier-free Na$_{252}$I and [2,8-$^3$H]ATP were purchased from DuPont NEN. Lysine 48-linked diubiquitin generated by recombinant E2$_{26K}$ (43) was the generous gift of Dr. Cecile Pickart (Johns Hopkins University). The monomer concentration of diubiquitin was determined spectrophotometrically from the extinction coefficient of free polypeptide (44). Homogenous wild type, di-, and mutant ubiquitins were radioiodinated by the chloramine-T procedure (45). Recrystallized BSA was obtained from Pentex and used for the preparation of 125I-rcmBSA (46). Rabbit reticulocyte-rich whole blood was generated by phenylhydrazine induction and used to generate fraction II (45). A portion of fraction II was used to prepare apparently homogenous E2$_{26K}$ and E1/E2-depleted fraction II (39, 47). Rabbit liver E1 was purified to apparent homogeneity by adapting reported affinity chromatography/fast protein liquid chromatography methods reported previously (47) and quantitated by the stoichiometric formation of ubiquitin [1H]adenylate (48). Homogenous native histone H2B (generous gift of Dr. Vaughn Jackson, Medical College of Wisconsin) and recombinant yeast CDC34 (UBC3) and RAD6 (UBC2) were those reported previously (49).

**Purification of Wild Type Ubiquitin—Bovine erythrocyte ubiquitin was purchased from Sigma as a lyophilized powder. Although previous lots of this polypeptide were sufficiently homogeneous for use without additional purification, recent lots have consistently contained several contaminating proteins that preclude accurate spectrophotometric quantitation of ubiquitin and obviate its direct use following radiodination. Therefore, commercial preparations were additionally purified by modification of published procedures (44). Commercial ubiquitin was dissolved in water to a final concentration of 5 mg/ml and then titrated to pH 4.5 (4 °C) with glacial acetic acid. Aliquots were applied to an HR 10/10 Mono S cation exchange fast protein liquid chromatography column (Pharmacia) equilibrated in 25 mM ammonium acetate (pH 4.5). Ubiquitin eluted as a single, symmetric peak at 0.29 ± NaCl within a linear gradient of 5 mM to 2 M NaCl. Ubiquitin-containing fractions were pooled and dialyzed overnight against distilled water using tubing (Spectra/Por 1, molecular weight cut off 12,000-14,000). The resulting dialysate was concentrated by lyophilization and then dissolved in a minimum amount of distilled water. Ubiquitin (>99% pure) was quantitated spectrophotometrically using an empirically determined 280-nm extinction coefficient of 0.166 ml/mg-cm (44). A portion of the homogeneous ubiquitin was used to prepare rmUb as described previously (49).

**Purification of Mutant Ubiquitins—Single-site mutagenesis of each lysine residue present within ubiquitin was accomplished by the polymerase chain reaction-based overlap extension method of Ho et al. (50) using the pPLhUb mutagenesis/expression plasmid described earlier (31). Following the final amplification step, the mutant polymerase chain reaction product was restricted with NdeI/SalI then ligated into NdeI/SalI-restricted pPLhUb. Generation of the predicted mutants was confirmed by DNA sequencing the complete ubiquitin coding region of the resulting pPLhUb constructs. Mutant ubiquitins were expressed in the Escherichia coli AR58 strain by heat induction and purified to apparent homogeneity without modification (51). Ubiquitin concentrations were determined spectrophotometrically as for wild type polypeptide. Typical yields were in the range 15 to 40 mg/liter for all mutants except UbK29R, which gave a consistent yield of approximately 0.5 mg/liter of culture. In all subsequent applications, all mutants displayed stabilities comparable to wild type polypeptide. In addition, all mutants exhibited CD spectra between 190 and 260 nm, indistinguishable from that of wild type ubiquitin (not shown).

**Ubiquitin-H2B Conjugation Assay—Initial rates of histone H2B monoubiquitination were measured for wild type and mutant ubiquitins as described (51). Briefly, various concentrations of wild type or mutant radiodinated ubiquitin (ca. 2-4 × 10$^6$ cpm/mg) were incubated at 37 °C in reactions of 25 μl, final volume, containing 50 mM Tris-CI (pH 8.0), 2 mM ATP, 10 mM MgCl$_2$, 1 mM dithiothreitol, 10 mM 2-mercaptoethanol, 15 μM ubiquitin, 1 IU creatine phosphokinase, 1 IU inorganic pyrophosphatase, 1 IU E1, and 20 nM E2$_{32K}$. In addition, all reactions contained 0.5 mg/ml BSA as a carrier protein to prevent adsorption of the enzymes to the reaction tubes. Reactions were quenched by addition of 25 μl of SDS sample buffer containing 3% (w/v) β-mercaptoethanol and then boiled for 5 min. Following SDS-PAGE resolution, the monoubiquitinated H2B band was excised and associated radiolabeled activity was determined by γ counting (43). Data were corrected for radioactivity present in an identical section of a control lane derived from an incubation performed in the absence of E1 and E2$_{32K}$. The incubation conditions were chosen to be E1 limiting, indicated by a linear dependence of initial rate on [E1], to kinetically isolate the ubiquitin-dependent activation step.

**Ubiquitin Linkage Specificity Assay**

The linkage specificity for multiquitin chain formation catalyzed by recombinant CDC34, RAD6, and E2$_{EPF}$ was determined in incubations similar to those for the kinetic assays with the exception that E1 and E2 concentrations were empirically adjusted to be rate-limiting with respect to E2 to kinetically isolate the step of multiquitin chain elongation, and histone was present only in the experiment testing RAD6 to serve as a substrate for conjugation (39). Each incubation was conducted under initial velocity conditions with 5 μM radiodinated wild type or mutant ubiquitin. Following resolution by 12% SDS-PAGE, the pattern of conjugates was visualized by autoradiography. Correction for slight differences in the specific activities of the radiodinated proteins was achieved by either normalizing for exposure at constant sample volume (Fig. 1) or adjusting the sample volume at constant exposure (Figs. 2-4).

**Proteasome Binding Assay—**The 26 S proteasome was partially purified from human erythrocytes by the method of Hough et al. (52). Proteolytic activity was monitored with the fluorogenic peptide N-succinyl-leucyl-leucyl-valyl-tyrosyl-7-amido-4-methylcoumarin. Fractions from the ATP-dependent protease hydrolysate of the proteasome were pooled and used in the direct conjugate binding assay of Deveraux et al. (37). Briefly, 25 μg of purified 26 S proteasome per lane was resolved by 10% SDS-PAGE and then electrothermally transferred to BA83 nitrocellulose (Schleicher and Schuell) (53). Excess nitrocellulose binding sites were blocked by incubation for 1 h with 50 mM Tris-CI (pH 7.5) containing 0.15% NaCl and 5% (w/v) powdered milk. The blots were then incubated for 1.5 h in 50 mM Tris-CI (pH 7.5) containing 0.1% NaCl, 25 mg/ml BSA, and 3 × 10$^8$ cpm/ml multiquitin chain prepared using 125I-ubiquitin and the indicated E2 isozyme. Nonspecifi-
E1-catalyzed forward reaction of E232K-mediated monoubiquitination. Whether introduction of the Lys3 point mutants have negligible effect on the E232K conjugation reaction. The autoradiogram of Fig. 1 represents the results obtained step supported by the isozyme. These results also indicate that the point mutants did not measurably affect the structure of ubiquitin, consistent with their retention of a native CD spectrum (not shown).

Confirmation of CDC34 linkage specificity. The linkage specificity for CDC34-catalyzed autoubiquitination was examined with radiolabeled wild type, reductively methylated, or mutant ubiquitins as described under “Materials and Methods.” Values represent the mean \pm S.D. from nonlinear least square fits of initial velocity versus ubiquitin concentration.

| K_m (M) | V_max (pmol/min) |
|---------|-----------------|
| Ub (wild type) | 0.74 \pm 0.19 | 0.07 \pm 0.02 |
| UbK6R | 0.70 \pm 0.23 | 0.07 \pm 0.02 |
| UbK11R | 2.02 \pm 0.50 | 0.07 \pm 0.01 |
| UbK22R | 1.39 \pm 0.42 | 0.08 \pm 0.03 |
| UbK29R | 1.20 \pm 0.32 | 0.11 \pm 0.03 |
| UbK33R | 0.51 \pm 0.14 | 0.07 \pm 0.01 |
| UbK48R | 1.29 \pm 0.17 | 0.08 \pm 0.02 |
| UbK63R | 1.30 \pm 0.16 | 0.08 \pm 0.02 |

Characterization of the Ubiquitin Mutants—To determine whether introduction of the Lys \rightarrow Arg point mutations into ubiquitin affected their folding or stability, the homogeneous polypeptides were tested for their ability to support the net E1-catalyzed forward reaction of E232K-mediated monoubiquitination of histone H2B under E1-limiting conditions (51). The initial rates for histone monoubiquitination followed hyperbolic kinetics with respect to ubiquitin concentration for each mutant from which values of K_m and V_max could be determined by nonlinear least squares fitting using the Enzfitter program. Table I summarizes values of K_m and V_max for wild type ubiquitin and the seven mutants. The value of K_m for wild type ubiquitin agrees with that determined previously by this kinetic method (51) and the intrinsic K_m measured under equilibrium conditions (54). Introduction of the point mutations into ubiquitin had only minor effects on the affinity of E1 for the polypeptides. For UbK11R, UbK48R, and UbK63R, the increase in K_m was greater than the combined standard error of the measurements. Although the overall effects are small, the results suggest there is weak interaction between the E1 active site and these lysine residues. This is in contrast to the marked effects of Arg \rightarrow Leu mutations on E1-ubiquitin binding (51). More important, the values of V_max are identical within experimental error among the eight proteins (Table I). Under the conditions of the assays, the rates were limited with respect to E232K at saturating ubiquitin, indicating that V_max reflects the step of E232K-catalyzed histone ubiquitination. Therefore, the point mutants have negligible effect on the E232K conjugation...
that E2EPF catalyzes multiubiquitin chain formation (56, 64).

Except for 125I-UbK11R, the other six arginine mutantsexhibit levels of the adducts, and suggests Lys-6 represents a specificity determinant for binding of CDC34 to the growing chain during elongation.

Determination of the Linkage Specificity for E2EPF and RAD6—The ubiquitin mutants were used in a manner similar to that of Fig. 1 to determine the linkage specificity for multiubiquitin chain formation catalyzed by recombinant E2EPF and RAD6. The autoradiogram of Fig. 2 illustrates the pattern of conjugates formed during E2EPF-catalyzed autoubiquitination (64). The pattern of bands is resolved to the E2EPF-Ub3 adduct, and the low steady state accumulation of the monoubiquitinated species reflects the highly processive nature of E2EPF autoubiquitination (56). As with the results of Fig. 1, the absence of a ladder of bands above that of E2EPF-Ub3 confirms that E2EPF catalyzes multiubiquitin chain formation (56, 64). Except for 125I-UbK11R, the other six arginine mutants exhibit patterns of multiubiquitin chain formation identical to that of wild type polypeptide (Fig. 2). The absence of multiubiquitin bands with 125I-UbK11R identifies this lysine as the exclusive site for chain elongation catalyzed by E2EPF.

Yeast RAD6 is not subject to autoubiquitination but does catalyze facile conjugation to core histones when used as model substrates (39). The autoradiogram of Fig. 3 represents the pattern of conjugates formed to histone H2B for each of the radioiodinated ubiquitin polypeptides. With wild type 125I-ubiquitin, a clear pattern of H2B conjugates is observed extending to the Ub3 adduct. The absence of discrete bands above that of the diubiquitin species in the presence of 125I-radiolabeled Ub confirms our earlier report that RAD6 is also capable of multiubiquitin chain formation (39). That these higher order bands are absent in the 125I-UbK6R lane confirms that multiubiquitin chain formation by RAD6 exhibits an exclusive linkage specificity requiring isopeptide bond formation through this residue (Fig. 3). This conclusion is supported by observation that the other arginine mutants support multiubiquitin chain formation indistinguishable from that of wild type polypeptide. These results confirm our earlier observations that multiubiquitin chain formation catalyzed by RAD6 does not require linkage to histone H2B in the reaction catalyzed by RAD6 (Fig. 3) and possess an average linkage number \( n \geq 7 \) specifically bound to a band of 50 kDa relative molecular mass (Fig. 4), previously identified as subunit 5 (37). At equivalent monomer concentrations, 125I-diubiquitin but not 125I-ubiquitin also bound to the same band (not shown), consistent with the binding specificity of subunit 5 in recognizing multiubiquitin chains (37). However, 125I-diubiquitin bound to subunit 5 only at monomer concentrations substantially greater than that of the CDC34 multiubiquitin chains, again in agreement with the increased affinity exhibited by this proteasome subunit for binding chains of \( n \approx 4 \) (37). Similarly, Lys-6-linked multiubiquitin chains formed to histone H2B in the reaction catalyzed by RAD6 (Fig. 3) and possessing an average linkage number \( n \geq 7 \) bound to a band having the same relative mobility (Fig. 4). Multiubiquitin chains linked through Lys-11 formed in the autoubiquitination of E2EPF (Fig. 2) and having an average linkage number \( n \geq 7 \) were also found to bind to the same subunit as those possessing Lys-48 and Lys-6 linkages (Fig. 4). In control studies (not shown), neither histone H2B nor E2EPF at an equivalent con-
FIG. 4. Subunit 5 of the 26 S proteasome binds alternatively linked multiubiquitin chains. Aliquots of partially purified 26 S proteasome (25 μg) were resolved by 10% SDS-PAGE and either stained with Coomassie Blue (left lane) or transferred to nitrocellulose and incubated with radiolabeled chains of the indicated linkage type as described under "Materials and Methods." Positions of subunit 5 and the 100-kDa putative proteasome T bands are indicated to the left. Positions of molecular weight markers are shown to the right.

Concentration had any effect on the binding of radiolabeled Lys-6- and Lys-11-linked chains, respectively, indicating that these conjugates did not bind to S5 through the target protein moiety to which they were conjugated.

During these studies, we consistently observed multiubiquitin chains possessing all three linkage specificities to associate with an additional protein band having a relative molecular mass of ca. 100 kDa (Fig. 4). This molecular weight is consistent with that of isopeptidase T, a ubiquitin-specific protease believed responsible for the disassembly of multiubiquitin chains and the subsequent utilization of monomeric polypeptide during the degradative cycle of the 26 S proteasome (57). This observation suggests isopeptidase T may possess a broad specificity for multiubiquitin chain disassembly. Studies are currently in progress to test this hypothesis with purified isopeptidase.

Differentially Linked Multiubiquitin Chains Compete For Binding to Subunit 5—The results of Fig. 4 suggest that subunit 5 is capable of binding multiubiquitin chains possessing different linkage specificities. However, the results do not preclude the alternative interpretation that the apparently homogeneous subunit 5 consists of mixed isoforms, each possessing specificity for binding chains containing different linkages. To test these alternative models, the ability of unlabeled diubiquitin and CDC34-bound Lys-48-linked chains to compete with radiolabeled Lys-48- and Lys-11-linked chains (n > 7) formed during the autoubiquitination of CDC34 and E2E6, respectively, was examined. Binding of radiolabeled chains was assessed by quantitating bound radioactivity within the S5 band by γ counting after correction for nonspecifically bound label contained on an equivalently sized portion of nitrocellulose from a parallel control lane containing no sample. The monomer concentration and weighted average linkage number for unlabeled Lys-48-linked chains to CDC34 were estimated from a parallel reaction in which 125I-ubiquitin was substituted for free polypeptide.

FIG. 5 shows that a 104-fold excess of diubiquitin results in only a 25% inhibition in binding of 125I-labeled Lys-48- or Lys-11-linked chains. This result confirms observations of Deveraux et al. (37) that subunit 5 exhibits a significantly diminished affinity for diubiquitin compared to Lys-48-linked chains of higher linkage number. In contrast, a 102-fold excess of unlabeled Lys-48-linked chains (n > 7) results in a 60–65% inhibition of both Lys-48- and Lys-11-linked radiolabeled chains. In parallel control studies, neither free CDC34 nor E2E6 at similar concentrations was capable of competing with its respective radiolabeled auto-multiubiquitin chains (not shown), ruling out the possibility that the apparent competition results from direct binding of the E2 isoforms to S5. Competition between unlabeled Lys-48-linked chains and labeled Lys-48- or Lys-11-linked chains suggests that a single subunit 5 species recognizes chains of alternate linkage specificity. In addition, subunit 5 must possess comparable affinities for Lys-48- and Lys-11-linked chains since unlabeled diubiquitin and Lys-48-linked chains result in similar degrees of inhibition for both homopolymer structures.

Effect of the Ubiquitin Mutants on Protein Degradation—The previous results indicate that RAD6 and E2E6 are capable of catalyzing E3-independent multibiquitination via linkages distinct from that of Lys-48 and that these alternate linkages are able to bind to the recognition subunit of the 26 S proteasome. Therefore, we were interested in examining whether these properties were reflected in the abilities of the E2 isoforms to support ubiquitin-dependent degradation. Prior work has shown that rabbit reticulocyte fraction II can be quantitatively depleted of endogenous E1 and E2 isoforms by passing the extract through a ubiquitin-linked affinity column (39, 64). Ubiquitin-dependent protein degradation can be reconstituted in the resulting depleted fraction II by supplementing with exogenous E1 and selected E2 isoforms (39, 64). Depleted fraction II was prepared as described under "Materials and Methods" and used to measure the initial rates of degradation of 125I-ratMBSA by the generation of trichloroacetic acid-soluble radioactivity (45). Preliminary experiments indicated that un-supplemented depleted fraction II exhibited no net ubiquitin-dependent degradation of the radiolabeled substrate in the presence of ATP nor was the rate of proteolysis increased when
only E1 was added to the incubations (not shown), consistent with previous findings (39, 64). In addition, depleted fraction II was unable to form conjugates with 125I-ubiquitin unless supplemented with E1 and an E2 isozyme (not shown).

Fig. 6 (panel A) illustrates rates of net ATP-dependent degradation observed with intact fraction II when supplemented with wild type or variant forms of ubiquitin. A significant decrease in degradative capacity is observed when rmUb is substituted for wild type polypeptide, indicating that the majority of proteolysis proceeds through degradative intermediates bearing multiubiquitin chains (33). That these chains predominantly contain Lys-48 linkages is demonstrated by the similar inhibition found with UbK48R (33). A 50% inhibition was consistently observed when incubations were supplemented with UbK11R. Inhibition by UbK11R within this context probably does not indicate formation of chains containing Lys-11 linkages within intact fraction II since complete inhibition is observed only with UbK48R; however, the data do not rule out the potential for chains bearing mixed linkages. More likely, inhibition by UbK11R reflects an effect of this mutant on either the rate of Lys-48-linked multiubiquitin chain elongation within the E3-dependent reaction or a diminished binding of Lys-48-linked UbK11R chains to subunit 5.

Profiles of net E2-dependent degradation obtained with depleted fraction II supplemented with recombinant E214K, the cognate isozyme for E3-dependent proteolysis within reticulocyte extracts, are qualitatively similar to those observed with intact extract (Fig. 6, panel B). That E214K supplementation of depleted fraction II is capable of quantitatively reconstituting the level of degradation observed with intact extract demonstrates that the bulk of ATP, ubiquitin-dependent proteolysis proceeds through an E214K-mediated pathway of conjugation. Panel C illustrates that RAD6 is competent to support protein degradation in depleted fraction II when supplemented with wild type ubiquitin, although the absolute rate of degradation is considerably attenuated (39). That RAD6 can complement degradation in fraction II is expected since this isoform is considered the yeast homolog of E214K for N-end rule-dependent degradation (58, 59). The relative efficacy of RAD6 in complementing degradation varied with different preparations of fraction II from which depleted extract was prepared (not shown), suggesting that additional component(s) required for this pathway also show preparation-dependent variability. Notable in the data of panel C is that degradation via a RAD6-dependent pathway requires formation of Lys-48-linked multiubiquitin chains rather than those linked by Lys-6 since degradation is inhibited to baseline values when the reactions are supplemented with either rmUb or UbK48R but not UbK6R. Therefore, RAD6 displays a change in multiubiquitin linkage specificity for E3-dependent conjugation compared to earlier results obtained in the absence of ligase (Fig. 3).

In contrast, depleted fraction II supplemented with recombinant E2EPF displays a dependence on formation of Lys-11-linked chains similar to that found in the E3-independent reactions of Fig. 2 (Fig. 6, panel D). The absolute ability of E2EPF to support degradation was considerably less than that found with intact extract (panel A) and varied with different preparations of fraction II (not shown). That E2EPF-catalyzed
degradation requires Lys-11-linked multiubiquitin chain formation is demonstrated by the complete inhibition of proteolysis observed with rmUb and Ubk11R but not Ubk48R. Therefore, unlike the results with RAD6, E2_{EPF} retains its Lys-11 linkage specificity within the context of E3-dependent protein degradation.

**DISCUSSION**

Pickart and Rose first resolved the E2 isoforms of reticulocytes and demonstrated their ability to catalyze E3-independent ligation of ubiquitin to a narrow range of model protein substrates (60). Subsequent studies have emphasized similarities in sequence of the core catalytic domains among members of the E2 family and distinctions in their participation in a variety of the regulatory phenotypes characteristic of ubiquitin-mediated protein degradation, their ability to conjugate ubiquitin to various test proteins, and their catalysis of multiubiquitin chain formation bearing discrete linkage specificities (39, 61). In the present studies, we have utilized Lys→Arg point mutants of ubiquitin to map the linkage specificities for multiubiquitin chain formation catalyzed by two members of the E2 family previously demonstrated to form Lys-48-independent chains and have characterized interactions between these structures and downstream components of the degradative pathway.

The autoradiogram of Fig. 2 demonstrates that recombinant human E2_{EPF} forms multiubiquitin chains exclusively through Lys-11 of the polypeptide since none of the other six lysine mutants significantly affects the pattern of radiolabeled conjugates resolved by SDS-PAGE. This is distinct from the absolute Lys-48 linkage specificity previously characterized for the analogous auto ubiquitination reaction of CDC34 (Fig. 1). In contrast, RAD6 exclusively forms Lys-6 linkages during chain elongation from the initial ubiquitin conjugated to histone H2B (Fig. 3). The distinct linkage specificities catalyzed by the three E2 isoforms probably arise from the core domain sequence differences since the peptide insertion within the core catalytic domain of CDC34 that has been proposed to account for its Lys-48 linkage specificity is absent in both E2_{EPF} and RAD6 (55). Available evidence suggests the carboxy-terminal extension domains present on RAD6 and CDC34 are not required for multiubiquitination since their deletion has little effect on the ability to support degradation or conjugation (29, 59). However, these observations may be a function of the cognate E3 isozymes examined. Conjugation of the initial ubiquitin moiety during CDC34-catalyzed autoubiquitination occurs intramolecularly between subunits of a transient homodimer (63); in contrast, the first ubiquitin ligated upon E2_{EPF} auto ubiquitination is within the monomeric polypeptide (62). The kinetic order for chain elongation is presently unknown for CDC34 and E2_{EPF}, although steric constraints imposed by the growing multiubiquitin chain suggests this step is intermolecular.

Multiubiquitin chains linked through Lys-48 yield a highly symmetric structure stabilized by defined packing interactions between the monomeric units (36). Multiubiquitin chains possessing novel linkages through Lys-6 or Lys-11 probably yield related but distinct symmetric structures stabilized by packing interactions unique from those found in Lys-48-linked chains. Retention of a defined linkage specificity during elongation of such novel structures must arise from complementary interactions between groups present on the growing multiubiquitin chain and the respective E2 isozyme. Moreover, fidelity in linkage specificity accompanying chain elongation probably requires the E2 to bind across more than a single ubiquitin unit and thus recognize a unique pattern of interaction sites. Such a model posits that each unique linkage specificity should be characterized by a distinct constellation of ubiquitin residues specifying these interactions. For CDC34 chain elongation, the minimum recognition unit requires three ubiquitin units in correct Lys-48 linkage. The hypothesis of discrete sites on ubiquitin directing linkage specificity is supported by our recent observations that mutation of ubiquitin residues directing the specificity for Lys-48 linkages during CDC34- and E2_{EPF}- catalyzed chain elongation have no effect on multiubiquitin chain formation by RAD6 and E2_{EPF}. Alteration in the kinetics of CDC34-catalyzed Lys-48 chain elongation revealed by the shift in steady state formation of CDC34-Ub₃ versus CDC34-Ub₂ intermediates (Fig. 1) suggests that Lys-6 contributes to define this linkage specificity either by direct interaction with the E2 or by stabilizing the incipient structure. The accumulated observations do not rule out an alternative interpretation that conjugation of the second ubiquitin in correct linkage during chain elongation directs formation of subsequent linkages by sterically blocking other available lysine residues present on the polypeptide. This interpretation appears unlikely since in the Lys-48 tetraubiquitin structure all lysine residues remain solvent exposed (36).

Because chains bearing different linkage specificities are expected to pack into unique structures, we were surprised to find that polymers of similar length linked through Lys-6, Lys-11, or Lys-48 bound with comparable apparent affinity to the S5 subunit of the 26 S proteasome (Fig. 4). Moreover, both Lys-48 and Lys-11 chains bound competitively to S5 (Fig. 5), precluding the existence of distinct isoforms of S5 able to discriminate between alternative structures. Either the unique structures expected for chains of different linkage pack to present the same ubiquitin surface residues for interaction with S5 or, more likely, the proteasome subunit contains subsets of interacting sites recognizing differentially linked chains. In either case, recognition of the alternatively linked chains by S5 must be of high affinity based on the nanomolar concentrations of these species used in Fig. 4. The competition experiments of Fig. 5 allow us to estimate the Kd for binding of Lys-48 chains to S5. If one reasonably assumes that labeled and unlabeled Lys-48-linked chains bind with equal affinity, then the 60% inhibition found for competition of 200 nM unlabeled chains with the 1.4 nM labeled chains present in the incubation predicts a Kd of 130 nM, expressed as monomer ubiquitin concentration. A linkage number n = 7 for both labeled and unlabeled chains requires an intrinsic Kd = 18 nM for chain binding. Similar calculations reveal that the 25% inhibition of Lys-48 chain binding by 15 μM diubiquitin requires an intrinsic Kd of 23 μM for the latter having a linkage number of n = 2.

This estimate probably represents a lower limit to the actual affinity since it is unlikely that the S5 subunit retains absolute native conformation following SDS-PAGE resolution, electrophoretic transfer, and binding to the nitrocellulose membrane. However, the magnitude of this estimated Kd suggests that the principal mechanistic effect of multiubiquitin chain formation is in increasing the affinity of the proteasome for target substrates over that of the unconjugated protein. This argument is consistent with the significant increase in rate of degradation for model substrates bearing multiubiquitin chains compared to those containing only single ubiquitin moieties (32). Enhanced affinity of the 26 S proteasome to bind multiubiquitin chain-linked substrates together with the marked ability of the

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3 We did not examine competition between Lys-6- and Lys-48-linked chains because of the technical problems associated with generating structures of the former linkage having a sufficiently high linkage number to make the experiment meaningful. This problem arises from the modest processivity of RAD6-catalyzed chain elongation (O. V. Baboshina and A. L. Haas, unpublished observation).
ligation pathway to recognize minute conformational changes arising from denaturation or exposure of discrete signals provides a formidable targeting mechanism for selective protein degradation within the cell.

We have previously shown that RAD6 and E2_{EPP} support ATP, ubiquitin-dependent degradation in E1/E2-depleted reticulocyte fraction II extracts when supplemented with exogenous activating enzyme (39, 64) and that RAD6 functions in the E3-independent targeting of 129-labeled histone H3 for degradation by purified human erythrocyte 26 S proteasome (32). The complementation studies of Fig. 6 confirm our earlier observations that RAD6 and E2_{EPP} support degradation and confirm in both cases that degradation preceding multiquitin conjugates intermediate is significantly attenuated in the presence of rmUb. Although RAD6 exhibits Lys-6 linkage specificity in E3-independent chain formation (Fig. 3), within depleted fraction II degradation proceeds exclusively through Lys-48 chains (Fig. 6, panel C). Conversely, E2_{EPP} retains the Lys-11 linkage specificity in both E3-independent chain formation (Fig. 2) and to the added depleted fraction II (Fig. 6, panel D). Therefore, the linkage specificity of these E2 isozymes is determined in part by the catalytic contribution of E3. Two lines of evidence suggest RAD6- and E2_{EPP}-dependent degradation within depleted fraction II proceeds through E3-catalyzed chain formation. First, both RAD6 and E2_{EPP} support formation of a heterogeneous distribution of 129-ubiquitin conjugates to endogenous proteins when added to E1-supplemented depleted fraction II that is similar to that observed for intact and E2_{E2E}-supplemented extract (not shown). Second, both RAD6 and E2_{EPP} exhibit extremely restricted substrate specificities for conjugation of exogenous substrates in the absence of E3 (39, 64) and are unable to catalyze a significant rate of rmB5A ligating (not shown). Therefore, these observations support and extend earlier observations that the multiquitin chain linkage formed with RAD6 is context specific with respect to the identity of the E3 involved.

At present, only Lys-48- and Lys-63-linked chains have been observed in vivo (35, 40). Formation of Lys-63-linked chains within yeast do not challenge the conclusion that Lys-48 chains represent the principal mechanism of degradative targeting since the former appear to serve a regulatory rather than proteolytic function (40). Moreover, the present observations indicate that detection of alternatively linked chains requires expression of the responsible E2/E3 pair. In the case of E2_{EPP}, this isoforms are abundant in only a limited number of cell types other than keratinocytes. 4 We are currently screening these cell lines for the presence of Lys-11-linked multiquitin chains. The functional significance of alternative chains is obscure at present, particularly since both Lys-11- and Lys-48-linked chains may equally competent to target degradation. Steady state concentrations of ubiquitin conjugates and therefore their rate of subsequent degradation by the 26 S proteasome depend on the relative rates of conjugation versus disassembly (47, 53). If chains of different linkage form or undergo disassembly at differential rates, then the presence of alternative structures may represent modulation of proteolysis for specific substrates or substrate subpopulations.

The present data provide additional evidence for the formation of multiquitin chains bearing linkage specificities distinct from that of Lys-48. In addition, these alternatively linked chains bind to the 26 S proteasome and, in the case of Lys-11- and Lys-48-linked chains, direct degradation by the complex. The results provide a framework for studies in progress assessing the role of various E2 isozenzymes in E3-dependent conjuga-

*4 C. A. Conrad and A. L. Haas, unpublished observations.*
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