Ubiquitin Conjugation by the Yeast RAD6 and CDC34 Gene Products

COMPARISON TO THEIR PUTATIVE RABBIT HOMOLOGS, E2\textsubscript{20K} AND E2\textsubscript{32K}*

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The recombinant yeast RAD6 and CDC34 gene products were expressed in *Escherichia coli* extracts and purified to apparent homogeneity. The physical and catalytic properties of RAD6 and CDC34 were similar but distinct from their putative rabbit reticulocyte homologs, E2\textsubscript{20K} and E2\textsubscript{32K}, respectively. Like their reticulocyte counterparts, RAD6 and CDC34 are bifunctional enzymes competent in both ubiquitin:protein ligase (E3)-independent and E3-dependent conjugation reactions. RAD6 and E2\textsubscript{20K} exhibit marked specificity for the conjugation of core histones and catalyze the processive ligation of up to three ubiquitin moieties directly to such model substrates. RAD6 differed from its putative E2\textsubscript{20K} homolog in exhibiting simple saturation behavior in the kinetics of histone conjugation and in being unable to distinguish functionally between core histones H2A and H2B, yielding identical values of $K_m$ (1.9 min\(^{-1}\)) and $K_m$ (20 $\mu$M). A slow rate of multiquitination involving formation of extended ubiquitin homopolymers on the histones was also observed with RAD6 and E2\textsubscript{20K}. Comparison of conjugate patterns among native, reductively methylated, and K48R ubiquitin variants demonstrated that the linkage between ubiquitin moieties formed by E2\textsubscript{20K} and RAD6 was not through Lys-48 of ubiquitin, the site previously demonstrated as a strong signal for degradation of the target protein. In contrast, CDC34 differs from its putative homolog, E2\textsubscript{32K}, in showing a specificity for conjugation to bovine serum albumin rather than to core histones. Both CDC34 and E2\textsubscript{32K} exhibit a marked kinetic selectivity for processive multiquitination via Lys-48 of ubiquitin. Calculations based on a model ubiquitin conjugation reaction indicated that E2\textsubscript{32K} and CDC34 preferentially catalyzed multiquitination over ligation of the polyprotein directly to target proteins. Formation of such ubiquitin homopolymers by E2\textsubscript{32K} and CDC34 suggests these enzymes may commit their respective target proteins to degradation via an E3-independent pathway.

The emerging roles of ubiquitin in cellular regulation require the covalent ligation of this 8.6-kDa polypeptide to free primary amino groups on various target proteins (most recently reviewed in Ref. 1). Ubiquitin conjugation was initially thought to proceed exclusively through a three-step mechanism discussed in detail elsewhere (2, 3) and summarized here in Equations 1–3. Ubiquitin-activating enzyme (E1)\(^{1}\) is responsible for the ATP-coupled activation of the carboxyl terminus of ubiquitin (Ub) to form a stable enzyme-bound ubiquitin thiol ester intermediate (Equation 1), actually present as a ternary complex composed of 1 eq each of the covalent thiol ester and its noncovalently bound precursor ubiquitin adenylate. Subsequent transfer of the E1-ubiquitin thiol ester to a specific sulfhydryl group on ubiquitin carrier protein (E2) yields a second intermediate (Equation 2) whose aminolysis is coupled to formation of the final isopeptide bond between ubiquitin and the target protein, catalyzed by ubiquitin:protein ligase (E3) (Equation 3).

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\begin{align*}
E_{1sh} + Ub + ATP & \rightarrow E_{1s,Ub} + AMP + PP_1 \\
E_{1s,Ub} + E_{2sh} & \rightarrow E_{1sh} + E_{2s,Ub} \\
E_{2s,Ub} + protein & \rightarrow E_{3} \rightarrow E_{2sh} + protein-Ub
\end{align*}
\]

Within cells, E2 exists as a family of related isozymes empirically defined by their ability to form ubiquitin thiol esters exclusively by transfer from the E1 ternary complex (4, 5). The rabbit reticulocyte enzymes have been resolved and purified to apparent homogeneity (3, 6). Preliminary kinetic data with these components under chemically defined conditions of rate-limiting [E2] indicate that two closely related isozymes, E2\textsubscript{14K44} and E2\textsubscript{14K53}, are the most active in supporting E3-dependent conjugation. Lower but significant rates of E3-dependent conjugation are also supported by reticulocyte E2\textsubscript{20K} and E2\textsubscript{32K} (3, 4).\(^{2}\) Target protein ligation also proceeds by an E3-independent pathway requiring only E1 and any of the E2 isozymes active in E3-dependent conjugation (3, 4, 7). The apparent yeast homologs of the two 14-kDa reticulocyte E2 isozymes have recently been cloned, and their inferred sequences have been reported but not characterized kinetically (8). Two other yeast E2 isozymes have also been cloned and expressed. The yeast RAD6 gene product is an ~24-kDa E2 required for DNA repair, induced mutagenesis, and sporulation (9). Yeast CDC34 is an E2 of ~35 kDa that is essential during mitosis for G1/S transit (10). It has

\(^{1}\) The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein (subscript denotes relative molecular mass in kilodaltons); E3, ubiquitin:protein ligase; RSA, bovine serum albumin; des-GGUb, native ubiquitin with carboxyl-terminal glycine dipeptide removed by limited tryptic digestion; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UbK48R, ubiquitin mutant with arginine substituted for Lys-48.

\(^{2}\) Reticulocyte E2\textsubscript{20K} is identical to the E2\textsubscript{32K} isozyme described by Pickart and Vella (6). The difference in relative molecular mass apparently results from variability in SDS-PAGE determinations between the laboratories.
been implicitly assumed, but never tested, that RAD6 and CDC34 support E3-dependent conjugation (12). A significant fraction of cellular ubiquitin exists as conjugates to a diverse set of proteins. Pooling studies with cell-free extracts, intact cultured cells, and tissues demonstrate that only a fraction of this conjugate pool is subject to degradation and that the entire pool continues cycles with its free constituent proteins through the action of isopeptidase(s) responsible for cleaving the ubiquitin-protein linkage, a process termed disassembly (reviewed in Refs. 13 and 14). Therefore, the apparent selectivity of this multienzyme degradative pathway for short-lived and abnormal proteins derives from both an intrinsic specificity for conjugation of such substrates and the relative partitioning of the resulting adducts between degradation and disassembly (13). Discrimination among alternative target proteins for ubiquitin conjugation is believed to depend in part on the identity of the N-terminal residue (15, 16) and steric factors such as relative protein motion and induced unfolding that affect the accessibility of target protein lysine residues (discussed in Ref. 13).

The degree of conjugate partitioning toward degradation in turn depends on the extent of target protein ubiquitination (17). Degradation is markedly favored by target protein multiquitylation, the formation of high molecular mass ubiquitin homopolymer conjugates (18). These multiquitin chains are linked exclusively through Lys-48 of the polypeptide (18), with no other sites of multiquitin linkage reported to date.

Although the rabbit and yeast E2 isozymes are reported to have qualitatively similar substrate specificities for E3-independent histone conjugation, we demonstrate here that these enzymes can be discriminated by their relative ability to form multiquitin chains and the linkage specificity between ubiquitin moieties of such chains. These data also show that RAD6 and CDC34 support E3-dependent conjugation in rabbit reticulocyte extracts.

**MATERIALS AND METHODS**

Ubiquitin was purchased from Sigma and used for the preparation of Affi-Gel-10 affinity columns (19). Ubiquitin was purified to homogeneity from bovine pancreas for all other applications (20). A portion of the latter ubiquitin was labeled with [14C] by the chloramine-T method (19) using carrier-free Na141I obtained from Amersham Radiochemicals. This same labeling procedure was utilized for iodination of the cloned K48R human ubiquitin mutant (Ubk48R) expressed and purified as described previously (18). A portion of the purified native ubiquitin was also used to prepare de-Gddlubiquitin by limited tryptic digestion (21). The [2,2'-3H]ATP and Na131I, used for E1 quantitation and ATP-PP, exchange kinetics, respectively, were purchased from Du Pont-New England Nuclear. Rabbit reticulocyte E1 and E2 isozymes were purified to homogeneity by a combination of affinity and high performance chromatographic procedures and were then quantitated by stoichiometric activity assays (3, 19). Homogeneous, native thymus core histones were those used previously (7). All other reagents were purchased from Sigma.

**Preparation of Reductively Methylated [3H]-Ubiquitin—Reductively methylated [3H]-ubiquitin was prepared from radiiodinated polypeptide since the converse steps of reductive methylation followed by iodination consistently yielded ubiquitin derivatives of low specific radioactivity and impaired ability to support E1-catalyzed ATP-PP, exchange. Briefly, 0.5 mg of freshly radiiodinated ubiquitin (~106 dpm/pmol) was incubated overnight at 37 °C in 50 mM NaHCO3, pH 9.0, containing 12 mM formaldehyde and 25 mM sodium cyanoborohydride. The sample was then dialyzed (3.5-kDa exclusion limit dialysis tubing) against 2 liters of distilled water. Quantitative derivatization of free and residual (99%) of ubiquitin was assayed by assay with 2,4,6-trinitrobenzensulfonic acid and fluorometrically by reaction with fluorescamine (7). Unlabeled reductively methylated ubiquitin was prepared in a similar manner using native polypeptide.

**Expression and Purification of Cloned Yeast RAD6 and CDC34 Gene Products—Cloned yeast RAD6 and CDC34 gene products were expressed in Escherichia coli strains harboring the appropriate plasmids (9, 10). Log-phase cells were grown at 37 °C in M9 medium supplemented with ampicillin (50 μg/ml) to an absorbance (600 nm) of 2.0 before induction by addition of isopropyl-β-D-thiogalactopyranoside (1 mM) to a final concentration of 3 mM. After induction for 2 h, cells were harvested by centrifugation, and washed once with 10 volumes of homogenization buffer (50 mM Tris-Cl, pH 7.5, containing 1 mM EDTA) and then centrifuged and resuspended in 7 volumes of the same. Cells were lysed in a French press and then incubated for 10 min at 37 °C in the presence of 10 μg/ml bovine pancreatic DNase I (2500 Kunitz units/mg of protein) before adjusting the incubation with 5 mM DTT. Following centrifugation for 10 min at 5000 × g, the resulting pellets were extracted twice with 7 volumes each of homogenization buffer. The supernatants from these washes were pooled with the original homogenate and centrifuged at 100,000 × g for 90 min.

Since the bacterial extracts contained a persistent proteolytic activity capable of inactivating ubiquitin, covalent affinity chromatography following addition of exogenous E1 was not attempted. Instead, yeast RAD6 and CDC34 enzymes were further purified by Mono Q HR 5/10 anion-exchange chromatography at 20 °C using a Pharmacia LKB Biotechnology fast protein liquid chromatography system (3). Elution position was assayed by 125I-ubiquitin thiol ester formation (3). Optimization of conditions and use of a discontinuous NaCl gradient allowed segregation of the conjugating enzymes from the endogenous contaminating proteins (Fig. 1). With repetitive injections, yeast RAD6 and CDC34 (upper and lower panels, respectively) consistently eluted within a total peak volume of 1 ml at 440 and 470 mM NaCl, respectively. Minor remaining contaminants were resolved by separation on an analytical Superose 12 gel exclusion column equilibrated with 50 mM Tris-Cl, pH 7.5, containing 50 mM NaCl and 1 mM DTT (data not shown). Peak fractions were concentrated to 0.5 mg/ml using a 5-ml capacity Amicon ultrafiltration cell fitted with a YM-5 membrane and were then quantitated by a stoichiometric activity assay requiring E1-dependent thiol ester formation to 125I-ubiquitin (3). Typical yields of RAD6 and CDC34 based on thiol ester formation were 950 and 230 pmol/liter of culture, respectively. Both cloned yeast E2 isozymes were stored at −80 °C. Under these conditions, the enzymes retained full activity for over 6 months and tolerated a moderate number of freeze/thaw cycles.

**Conjugation Assays—**Conjugation assays were performed at 37 °C as described previously (3, 7) in incubations containing 50 mM Tris-Cl, pH 7.5, 2 mM ATP, 10 mM MgCl2, 0.5 mM DTT, 20 IU/ml yeast inorganic pyrophosphatase, and the indicated concentrations of 125I-ubiquitin and E1 and E2 isozymes. The resulting conjugates were resolved by SDS-PAGE. After autoradiography, conjugate formation was quantitated by cutting bands from the dried gel and determining covalently bound label. The absolute content of ubiquitinated histone determined by γ-counting was calculated from the specific activity of the radiiodinated ubiquitin (8–10 × 105 cpm/pmol). Saturation kinetics were determined only for histone monoubiquitination to avoid corrections required for subsequent partitioning to higher order adducts as discussed elsewhere (7). A similar approach was used for quantitation of heterogenous conjugates formed in E3-catalyzed reactions with the exception that the complete lanes of conjugates was counted (3). In these latter studies, crude E3 and endogenous protein substrates were contained in a dialyzed 30% ammonium sulfate precipitate of Fraction II, as described elsewhere (3).

**RESULTS**

**Putative Reticulocyte and Yeast Homologs Differ in Electrophoretic Mobility—**The qualitative similarity of band patterns by SDS-PAGE for E2 isozymes isolated from rabbit reticulocytes, plants, and yeast has been noted previously (3, 9–11). These observations constitute evidence for a family of evolutionarily conserved E2 isozymes; however, it does not follow
a priori that similarity in pattern constitutes similarity in function. Although the yeast and reticulocyte isozymes bear somewhat similar net charges, based on their elution positions from Mono Q anion-exchange columns (Fig. 1) (3), the proteins differ in other physical properties. Purified RAD6 and CDC34 do not exhibit the same relative molecular mass as their putative reticulocyte homologs when resolved by conventional SDS-PAGE (data not shown), and the electrophoretic mobilities are not similar for the corresponding 125I-ubiquitin thiol esters of the presumed homolog pairs when resolved by nonreducing SDS-PAGE to preserve this labile linkage (Fig. 2) (3, 19). These differences are not mobility artifacts since E1-ubiquitin thiol ester, required for loading the respective E2 intermediates (3), has identical mobilities in lanes 2–5 of Fig. 2. Although resolution remains principally a function of relative molecular mass, the electrophoretic mobilities observed under the latter conditions are also affected by residual structure contributed by stable unfolding intermediates, as has been discussed previously (3, 19). In addition, the E220K isozyme forms two ubiquitin thiol esters, as reported earlier (3), whereas RAD6 forms only a single thiol ester, consistent with the reported cysteine content of the latter (9). These observations taken together suggest structural similarity (but not identity) between the putative homologs.

**Yeast RAD6 and CDC34 Differ in Substrate Specificity—** Rabbit reticulocyte E220K and E232K exhibit comparable $k_{cat}$ and $K_m$ values for E3-independent conjugation of ubiquitin to histones H2A and H2B (7). In contrast, RAD6 and CDC34 markedly differ in their ability to ligate ubiquitin to these core histones (Fig. 3). Cloned CDC34 is significantly less active than RAD6 under identical conditions for the ubiquitination of either histone H2B (lanes 1–4) or H2A (lanes 5 and 6). Apparent initial velocities for monoubiquitination by RAD6 and CDC34 were proportional to the rates of subsequent ubiquitin ligation. As was reported with E220K and E232K (7), RAD6 catalyzes the facile ligation of three ubiquitins/histone molecule (Fig. 3). At a 3-fold higher enzyme concentration, CDC34 also catalyzes a marginal level of ligation beyond uH2B (lane 3), indicating that the apparent lack of conjugation at the lower concentration represents a rate effect. At the higher concentration, additional ubiquitination by RAD6 beyond uH histone is pronounced (lane 4).

Since concentrations of RAD6 and CDC34 are determined by a functional stoichiometric assay involving thiol ester formation with 125I-ubiquitin, the results of Fig. 3 are not a consequence of different fractions of active enzyme. Separate control experiments verified that E1 was not rate-limiting for any of the incubations (data not shown). As noted previously (7), the apparent smearing of conjugate bands probably reflects mobility differences resulting from slight changes in Stokes radii for conjugation at alternative sites on the histones, an effect particularly pronounced for small target proteins. By this reasoning, RAD6 appears to exhibit qualitatively greater heterogeneity in its sites for ubiquitin ligation (Fig. 3) than noted previously for either reticulocyte E220K or E232K (7).

*Fig. 1. Mono Q anion-exchange purification of cloned RAD6 and CDC34.* Post-ribosomal E. coli extracts from clones harboring expression plasmids for either RAD6 (upper panel) or CDC34 (lower panel) were resolved by high performance liquid chromatography using a Mono Q HR 5/10 anion-exchange column at 20°C. The column was equilibrated at a flow rate of 1 ml/min with 50 mM Tris-Cl, pH 7.5, containing 0.5 mM DTT. After the absorbance due to nonadsorbed protein returned to baseline, bound protein was eluted with the indicated discontinuous NaCl gradient. Elution positions for the respective cloned proteins and the corresponding salt concentrations are indicated by arrows.

*Fig. 2. Comparison of electrophoretic mobilities for 125I-ubiquitin thiol esters between putative E2 homologs.* Thiol esters to radioiodinated ubiquitin were formed at 37°C in incubations of 50 μl containing 2 pmol each of E1 and the indicated homogeneous E2 isozymes and were then resolved by SDS-PAGE under nonreducing conditions (7). Lane 1, 125I-ubiquitin alone; lane 2, rabbit reticulocyte E220K; lane 3, yeast RAD6; lane 4, rabbit reticulocyte E232K; lane 5, yeast CDC34. Migration positions for 125I-ubiquitin ($^{125}$I-Ub) and the corresponding E1 thiol ester, required for E2 loading, are indicated to the right.

The RAD6-catalyzed conjugation of ubiquitin was relatively specific for histones since barely detectable levels of conjugation to alkaline-denatured (incubation at pH 13 for 10 min) low molecular mass basic proteins such as horse heart cytochrome c or egg white lysozyme were observed in the presence of high concentrations of enzyme (data not shown). Cloned CDC34 exhibited a broader substrate specificity and was...
Fig. 3. RAD6 and CDC34 differ in substrate specificity for histone conjugation. Conjugation of \(^{125}\)I-ubiquitin to either histone H2B (lanes 1-4) or H2A (lanes 5 and 6) was allowed to proceed for 20 min at 37°C in a final volume of 25 μl as described under "Materials and Methods." Accumulation of ubiquitin adducts was linear over the incubation time chosen. The products were resolved by SDS-PAGE and visualized by autoradiography (3, 7). Incubations contained a 15 μM concentration of the indicated histone, 20 nM El, and the following concentrations of yeast conjugating enzyme: 20 nM CDC34 (lane 1), 20 nM RAD6 (lane 2), 60 nM CDC34 (lane 3), 60 nM RAD6 (lane 4), 20 nM CDC34 (lane 5), and 20 nM RAD6 (lane 6).

Fig. 4. CDC34 catalyzes E3-independent conjugation of ubiquitin to BSA. Conjugation reactions containing 50 nM CDC34 were carried out as described for Fig. 3 except that incubations contained either 150 nM (lanes 1 and 2) or 30 nM (lanes 3-5) homologous reticulocyte El. In the incubations, BSA present as carrier to prevent nonspecific adsorption of the conjugating enzymes and histones was at a concentration of 2 mg/ml (30 μM) in lanes 1 and 2 or of 0.4 mg/ml (6 μM) in lanes 3-5. In addition, incubations contained no added histone H2A (lanes 1 and 3), 2 μM histone H2A (lanes 2 and 4), or 20 μM histone H2A (lane 5).

Fig. 5. Reciprocal plot of histone concentration dependence for RAD6-catalyzed monoubiquitination. Initial rates of histone H2A (○) or H2B (○) monoubiquitination were determined in the presence of a 20 nM concentration each of cloned yeast RAD6 and rabbit reticulocyte El (7). Further evidence against contaminating E3 was the absence of conjugation to BSA when E2\(_{14K}\), the cognate E3-dependent ubiquitin carrier protein (3, 7), was substituted for CDC34 in other control incubations (data not shown). Cloned RAD6 was incapable of conjugating ubiquitin to either BSA or other non-histone proteins in repeated trials with different preparations (data not shown).

Although RAD6 is relatively specific for histones, this E2 isozyme is incapable of discriminating kinetically between histones H2A and H2B, as shown by the superimposable reciprocal plots for RAD6-catalyzed monoubiquitination of these core histones (Fig. 5). Graphically determined \(k_{un} values for the two histones averaged 1.9 min\(^{-1}\), an order of magnitude greater than that previously found for either reticulocyte E2\(_{20K}\) or E2\(_{23K}\) (6, 7). Conjugation to both histones exhibited \(K_{m} values of 20 μM, values somewhat larger than those found with the reticulocyte E2 isozymes. The lack of discrimination between histones H2A and H2B by RAD6 was in contrast to reticulocyte E2\(_{20K}\) and E2\(_{23K}\), both of which show a consistently greater affinity for histone H2A than for histone H2B (7). In addition, the single-site hyperbolic kinetics of RAD6 (Fig. 5) are distinct from the complex kinetics of E2\(_{20K}\), for which a saturable catalyzed binding process is superimposed over a slower second-order reaction (7).

Reticulocyte E2\(_{20K}\) and E2\(_{23K}\) Differ in Their Ability to Multiquitinate Histones—The autoradiograph of Fig. 6 demonstrates that E2\(_{20K}\) and E2\(_{23K}\) can be distinguished by their linkage specificity for multiquitination. This conclusion is based on comparing the patterns of conjugates generated with native \(^{125}\)I-ubiquitin to those formed with reductively methylated \(^{125}\)I-ubiquitin, a derivative for which multiquitination is not possible. Conjugation to Lys-48 of ubiquitin was tested by substituting \(^{125}\)I-UbK48R for native ubiquitin. Preliminary studies verified that native, reductively methylated, and K48R ubiquitin variants were functionally indistinguishable in supporting El-catalyzed ATP-PP exchange (Table I). As demonstrated below, this conclusion also holds.
panels) with their parallel controls are illustrated. Linkage type was conducted for 20 min at 37°C.

Ubiquitin (upper right panels), H4 (lane 3). Although not shown, histone H2A exhibited substrate properties identical to those of histone H2B in each of the experiments, as reported previously (7). The upper-most band in each lane is a noncovalent mobility artifact resulting from carrier BSA present in the incubations as noted earlier (7). Exposure times have been adjusted to correct for slight differences in specific radioactivity among the three forms of ubiquitin.

**TABLE I**

| Ubiquitin variant | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) |
|-------------------|------------|---------------------|
| Native            | 0.15       | 3.5                 |
| Reductively methylated | 0.33       | 2.9                 |
| K48R              | 0.10       | 4.7                 |

Kinetic comparison of native, reductively methylated, and K48R ubiquitins in supporting E1-catalyzed ATP-PP, exchange

The concentration dependence for support of ubiquitin-activating enzyme-catalyzed ATP-PP, exchange by homogeneous native, reductively methylated, and K48R mutant ubiquitins was determined in parallel using 10 nM E1 as described previously (22). To simplify interpretation, incubations also contained 1 mM AMP so that rates only reflected exchange in the absence of enzyme-bound ubiquitin thiol ester (Ref. 22, Step 1). Concentrations of reductively methylated and K48R polypeptides were determined by the method of Lowry et al. (33) using native ubiquitin as standard.

for subsequent conjugation since the relative band density for the monoubiquitin adduct is unchanged for each species.

At a concentration of 20 nM, $E_{220K}$ can conjugate three ubiquitin moieties to histones H2B and H3 (Fig. 6, left panel), consistent with results from Fig. 3. Higher order conjugates to these two histones are detected following much longer exposure (data not shown). As noted previously (7), histone H4 is an inherently poor substrate for conjugation by both reticulocyte E2 isozymes, principally yielding a monoubiquitin adduct (left panels). On longer exposure, higher order conjugates to histone H4 are observed in roughly the same relative proportions as observed for histones H2B and H3, indicating that the apparent inability to form conjugates of higher linkage number than uH4 is a simple rate effect. The first three ubiquitins attached are ligated directly to the target histones since the conjugate pattern for each histone is unchanged when reductively methylated $^{125}$I-ubiquitin is substituted (upper right panel). By similar reasoning, longer exposures revealed, for all four histones, that conjugates of linkage number greater than uH4 were principally composed of multibuquitin adducts (data not shown).

The incubation time in Fig. 6 was chosen to be within the linear velocity region for conjugate formation; therefore, the radiographic intensities of the three conjugate bands are proportional to their rates of formation. In parallel studies (7), initial rates of formation for the three principal conjugate species were determined for histone H2B by quantitating the respective amounts of $^{125}$I-ubiquitin bound. For the conditions present in Fig. 6, the observed initial rates of ligation for each conjugate species formed by $E_{220K}$ are listed in Table II. Observed rates of formation ($v_{obs}$) for addition of the second and third ubiquitins are both ~10-fold lower than those for addition of the first. Since [histone H2B] is within the V/K regions of $E_{220K}$ (7), the observed rate for addition of ubiquitin should be linear with respect to concentration of the acceptor substrate, assuming that the three sites for histone conjugation are equally reactive. Therefore, the predicted rate for addition of the nth ubiquitin ($v_{calc}$) is equal to $k[E_{220K}] [u]$ for histone H2B. Predicted values for these initial rates are also summarized in Table II. Comparison of the observed to calculated rates reveals modest catalysis for addition of the second ubiquitin (4-fold over theoretical) compared to ligation of the third moiety (~60-fold over theoretical). The data of Table II constitute quantitative evidence for modest processivity of histone polylubiquitination by $E_{220K}$.

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Significant conjugation to histones H2B and H3 in excess of the three-ubiquitin adduct is observed with 20 nM $E_{232K}$ (Fig. 6, upper left panel), as reported previously (6, 7). However, these high molecular mass adducts are almost exclusively multibuquitin homopolymers since conjugation again occurs at only three sites on the parent histones in the presence of reductively methylated $^{125}$I-ubiquitin (upper right panel). The observed increase in the levels of monoubiquitin adducts is expected when subsequent multibuquitination is blocked. The kinetically favored linkage for the multibuquitin homo-

**FIG. 6. Characterization of ubiquitin linkages formed by $E_{220K}$ and $E_{232K}$.** Isopeptide ligase-independent histone conjugation was conducted for 20 min at 37°C in standard incubations (7) containing a 20 nM concentration each of E1 and the indicated reticulocyte E2. Two separate experiments (upper versus lower panels) with their parallel controls are illustrated. Linkage type was distinguished by including in the incubations a 5 μM concentration of native $^{125}$I-ubiquitin (left panels), reductively methylated $^{125}$I-ubiquitin (upper right panels), or $^{125}$I-UbK48R (lower right panels). The following histones were present at 15 μM: H2B (lane 1), H3 (lane 2), and H4 (lane 3). Although not shown, histone H2A exhibited substrate properties identical to those of histone H2B in each of the experiments, as reported previously (7). The upper-most band in each lane is a noncovalent mobility artifact resulting from carrier BSA present in the incubations as noted earlier (7). Exposure times have been adjusted to correct for slight differences in specific radioactivity among the three forms of ubiquitin.

**TABLE II**

Pro obsessivity of histone conjugation

Initial rates of histone H2B conjugation were determined at 37°C under conditions of rate-limiting $E_2$ in incubations containing 50 mM Tris-Cl, pH 7.5, 0.2 mg/ml carrier BSA, 1 mM ATP, 5 mM MgCl$_2$, 1 mM DTT, 1.5 μM histone H2B, 40 nM E1, and 20 nM either $E_{220K}$ or $E_{232K}$.

| Species | $v_{obs}$ (pmol/min) | $v_{calc}$ (pmol/min) | $v_{calc}/v_{obs}$ |
|---------|----------------------|-----------------------|-------------------|
| $E_{220K}$ |                        |                       |                   |
| uH2B    | 7.5 × 10$^{-1}$       | 1.8 × 10$^{-1}$       | 4                 |
| u2H2B   | 6.6 × 10$^{-2}$       | 1.7 × 10$^{-2}$       | 3900              |
| uH2B    | 5.9 × 10$^{-2}$       | 1.2 × 10$^{-2}$       | 3700              |
| $E_{232K}$ |                        |                       |                   |
| uH2B    | 7.7 × 10$^{-1}$       | 7.7 × 10$^{-1}$       | 1                 |
| u2H2B   | 1.6 × 10$^{-2}$       | 1.7 × 10$^{-2}$       | 9300              |
| uH2B    | 3.4 × 10$^{-3}$       | 1.5 × 10$^{-3}$       | 1800              |
| uH2B    | 3.0 × 10$^{-3}$       | 5.1 × 10$^{-3}$       | 3700              |
polymer chains formed by E2_{20k} is between the carboxyl terminus and Lys-48 of the adjacent molecule. This conclusion is supported by the loss of conjugates greater than u3histone when 125I-UBK48R is substituted for native polypeptide (compare lower two panels). Monoadducts with the mutant ubiquitin are detectably greater than those with native polypeptide since multiubiquitination is again blocked. In replicates of experiments such as that in Fig. 6, there was consistently little change in u3histone levels with UbK48R; however, u3histone levels were generally less than half those found with native ubiquitin. Comparing u3histone intensities between parallel incubations containing either reductively methylated or K48R ubiquitin variants (right panels), the third ubiquitin ligated by E2_{23k} is predominantly in multiubiquitin linkage, only about half of which is to Lys-48. This is in contrast to the near-absolute specificity for ligation to Lys-48 in conjugates of linkage number greater than u5. These observations suggest some site degeneracy in forming the first multiubiquitin bond. As expected, conjugate patterns formed by E2_{20k} were unaltered by 125I-UbK48R (lower panels) since multiubiquitination by this isozyme; in addition, the data suggest that E2_{20k} is more active in conjugating ubiquitin to Lys-48 of a second ubiquitin than in the initial ligation of polypeptide to target histone.

RAD6 and CDC34 Differ in Their Rates and Linkages of Multiubiquitination—Fig. 7 represents an autoradiograph comparing RAD6 conjugation to core histones using native, reductively methylated, or K48R mutant radioiodinated ubiquitin. Unlike E2_{23k}, the formation of u3histone by RAD6 is via multiubiquitination when the pattern for native is compared to reductively methylated ubiquitin. However, this multiubiquitin linkage is not to Lys-48 since substitution with UbK48R has no effect on the conjugate pattern. Similar results have been reported (23) for conjugation of unlabeled ubiquitin to 125I-histone H3. Although rates of conjugation were not analyzed, it is clear from Fig. 7 that polyubiquitination of the histones by RAD6 is processive. Also obvious is that at equivalent concentrations, RAD6 is much less effective in multiubiquitination than E2_{23k} (Figs. 6 and 7).

In contrast, CDC34 rapidly multiubiquitinated BSA present as carrier protein within the incubations since only a single major band is observed with reductively methylated ubiquitin (Fig. 8, lanes 1 and 2 and lanes 3 and 4, respectively). Because only minor levels of monoaduct accumulate with native polypeptide, multiubiquitination by CDC34 must be highly processive. Most of the ubiquitin-ubiquitin bonds formed to BSA in Fig. 8 are via Lys-48 since only the monoaduct is observed autoradiographically (data not shown). From these bands correspond to the faint lowest bands with native 125I-ubiquitin and also were present using either reductively methylated or K48R 125I-ubiquitin.

RAD6 and CDC34 Support E3-dependent Ubiquitin Conjugation—The reticulocyte E2_{48k} isozymes (3, 6) and the corresponding yeast UBC4 and UBC5 isozymes (8) are quantitatively the most active E2 species in supporting E3-dependent ubiquitin conjugation. We have previously demonstrated (3) that E2_{20k} and E2_{23k} also support significant rates of E3-dependent ligation relative to that of the cognate 14-kDa species. In the presence of pure E1 and a crude reticulo-

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5 Recent studies have shown that des-GGUb is capable of E1-catalyzed activation based on rates of ATP-PP exchange (A. L. Haas and F. B. Rebeck, un unpublished observations). The k_{cat} for exchange is only ~2-fold lower for des-GGUb than for native polypeptide. The apparent inactivity of des-GGUb results from a 25-fold greater than that for native polypeptide. In model multiubiquitination reactions using des-GGUb as substrate, the rate of direct des-GGUb ligation is negligible.

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**Fig. 7. Characterization of ubiquitin linkages formed by RAD6.** The linkages of conjugates formed to core histones by yeast RAD6 (20 nM) were identified in incubations identical to those described for Fig. 6 using the indicated 125I-ubiquitin. Lane 1, histone H2A; lane 2, histone H2B; lane 3, histone H3; lane 4, histone H4. Exposure times were adjusted as described for Fig. 6. Ub, ubiquitin; RM-Ub, reductively methylated ubiquitin.
Ubiquitin Ligation by RAD6 and CDC34

Fig. 8. Characterization of ubiquitin linkages formed by CDC34. Incubations were conducted identically to those described for Fig. 4 in the absence (lanes 1, 2, and 5) or presence (lanes 2, 4, and 6) of ATP. Conjugates to exogenous BSA (2 mg/ml) were compared for native [15S] ubiquitin (lanes 1 and 2), reductively methylated [15S]-ubiquitin (lanes 3 and 4), or [15S]-UbK48R (lanes 5 and 6). Trace amounts of low molecular mass labeled contaminants are indicated to the right. Exposure times were adjusted as described for Fig. 4.

Fig. 9. Concentration dependence of E2 in supporting E3-catalyzed conjugation. Initial rates for approach to steady state of ubiquitin conjugation were determined as described previously (3, 7) using crude reticulocyte E3, 20 nM E1, 10 μM [15S]-ubiquitin, and the indicated concentrations of either reticulocyte E214K (●) or RAD6 (○). Similar experiments were conducted for the other E2 isozymes. The resulting Vmax and Km values are summarized in Table III.

The effect of E2 concentration on the initial velocity for E3-dependent conjugation was examined for each of the isozymes. In each case, hyperbolic kinetics were observed, as shown by the representative linear reciprocal plots for yeast RAD6- and reticulocyte E214K-supported E3-dependent conjugation shown in Fig. 9. In preliminary range studies, linearity was maintained over a broader concentration range than illustrated in Fig. 9. Even at the lowest E2 concentration, there was no effect on rate when [E1] was doubled above that used in Fig. 9 (data not shown). This control experiment rules out the results of Fig. 9 arising by a change in the rate-limiting step from E2 to E1 at the lowest concentrations of the former.

Hyperbolic kinetics with respect to E2 isozyme concentration are most consistent with an equilibrium step involving the binding of E2-ubiquitin thiol ester to E3. Table III summarizes the Km values for this binding step and the Vmax values for the respective isozyme-supported E3-dependent rates of conjugation. At saturating E2-thiol ester concentrations, Vmax represents a rate-limiting E3-catalyzed step since the apparent maximum velocity was proportional to the concentration of crude E3 (data not shown). The E2 isozymes tend to segregate into two classes (Table III). One class, represented by E214K and RAD6, exhibits very similar Km values and maximum rates of E3-dependent conjugation. The remaining three isozymes exhibit significantly lower Km and Vmax values. The kinetic similarity between E214K and RAD6 is surprising and likely reflects marked structural and sequence similarity between the two isozymes, as is generally characteristic of this family of E2 isozymes (24).

DISCUSSION

Significant homology among the reported sequences of yeast (8–10, 24), human (25), and plant (26) E2 isozymes has in some instances been used to infer identity and function among these closely related proteins without consideration for either substrate specificity or catalytic activity. In an effort to address the latter points, this study represents the first detailed characterization of the yeast RAD6 and CDC34 E2 isozymes since their identification as components of ubiquitin ligation (9, 10). In addition, this is the first instance in which conjugating enzymes have been compared from diverse species. This study reveals similarities and differences between the yeast and rabbit enzymes and illustrates the dangers inherent in inferring function and identity among these closely related proteins in the absence of rigorous characterization.

Yeast RAD6 and its putative reticulocyte E220K homolog share marked specificity for the ubiquitination of histones, as noted previously (4, 6, 7, 9) and more fully characterized here. Conjugation is highly processive for both enzymes and kinetically favors ligation directly to the target histones over chain extension to form mult ubiquitin homopolymers (Figs. 6 and 7). Sung et al. (11) have reported RAD6 to ligate up to seven ubiquitins to histones. Our results show that at least three of these ubiquitins are bound directly to the target protein since conjugation occurs at two major sites and a minor third site on the histones tested. Low rates of mult ubiquitination, generating conjugates of higher linkage number, are observed for E220K and RAD6. Unlike the results found with E220K and CDC34, the pattern of adducts formed by E220K and RAD6 using ubiquitin variants demonstrates that the mult ubiquitin

Table III

| E2 isoform | Km (nM) | Vmax (pmol/min) |
|------------|---------|-----------------|
| E214K      | 25      | 9.1 (100)       |
| RAD6       | 27      | 3.6 (40)        |
| E220K      | 7       | 1.2 (13)        |
| CDC34      | 4       | 0.6 (7)         |
| E220K      | 11      | 0.9 (10)        |

*Values in parentheses are percentages.
linkage is not through Lys-48 (Figs. 6 and 7). Qualitative differences in the conjugate patterns and relative extent of mult ubiquitination between these latter isozymes can be accounted for by rate arguments reflecting the 10-fold greater kcat for RAD6 over its putative homolog (this study and Ref. 7). It is unclear whether homopolymer formation by RAD6 occurs at a specific site or randomly over all accessible lysines of ubiquitin; however, sequestering of the total conjugate pool among subpopulations differing in their mult ubiquitin linkage would be a particularly effective means of dictating specificity for the fate of such adducts. Interestingly, RAD6-catalyzed mult ubiquitina adducts of 17p-histone H3 are better substrates for degradation by the reticulocyte high molecular mass 2S protease than either the corresponding monoubiquitin adducts formed with reductively methylated ubiquitin or Lys-48 mult ubiquitin conjugates formed by E220K (23).

In spite of the apparent similarities between RAD6 and E220K, the two isozymes differ in several important respects. Their amino acid compositions must be sufficiently different to affect net charge on the proteins and their corresponding elution positions during high resolution Mono Q fast protein liquid chromatography (Fig. 1) (3). The most functionally important difference is that RAD6 forms only a monoubiquitin thiol ester intermediate, as predicted by its inferred sequence (9), whereas E220K supports two such intermediates (Fig. 2) (3). Distinct stoichiometries may account for the simple hyperbolic kinetics in histone conjugation by RAD6 (Fig. 5), catalyzed at a single thiol ester site, compared to the complex biphasic kinetics of histone ligation by E220K (7), representing the sum of a rapid saturable process at one thiol ester site and a slower second-order reaction at the second site.

Reticulocyte E220K and its putative yeast homolog (CDC34) exhibit minor differences in relative molecular masses, net charge, and ubiquitin thiol ester electrophoretic mobility (Figs. 1 and 2). Both mediate a highly processive mult ubiquitination of target proteins to form extended ubiquitin chains linked at Lys-48 (Figs. 6 and 8). The principal difference between E220K and CDC34 lies in their apparent ability to utilize histone on BSA as model substrates, respectively (Figs. 4 and 8). This observation is significant since CDC34 and RAD6 both contain polyacidic carboxyl-terminal amino acid sequences that have been proposed (9-11) as an essential binding site conferring specificity for histone ligation. Loss in ability of RAD6 to catalyze transfer of ubiquitin thiol ester to histones upon deletion of this polyacidic tail has been considered proof of this hypothesis (11). The marked conjugation of BSA by CDC34 suggests that a polyacidic tail is necessary but not sufficient to direct specificity for histone conjugation.

Indeed, the true substrate specificity of E220K and CDC34 appears to be in catalyzing target protein mult ubiquitination. This is most dramatic with BSA ubiquitination by CDC34, in which uBSA and u2BSA barely accumulate because of rapid subsequent chain elongation (Fig. 8). Rate measurements of histone mult ubiquitination by E220K (Table II), for which we can only set a lower limit to the catalytic acceleration, indicate that chain formation by this isozyme, and CDC34 by inference, is the kinetically preferred pathway for conjugation. Since mult ubiquitination at Lys-48 of the polypeptide preferentially targets proteins involved in DNA repair and the associated DNA damage-induced mutagenesis (28), suggesting that RAD6 is a multifunctional repair enzyme. Two more recent observations (29) demonstrate that ubiquitin conjugation is required for all three functions. First, mutation of the single Cys-88 of RAD6 to alanine or valine blocks E1-dependent thiol ester formation and histone conjugation. Second, yeast strains carrying either Cys-88 mutant are equivalent to that of the RAD6 null allele. Based on these observations and those presented here, we propose that DNA repair and the associated DNA damage-induced mutagenesis are mediated by a RAD6-supported E3-dependent conjugation mechanism.

One may speculate that the E2 isozymes normally function both in the E3-independent conjugation of a limited subset of specific proteins and in a more general E3-dependent pathway with a broader range of potential substrates. Monoubiquitination may proceed through the kinetically favored E220K-supported E3-dependent conjugation (Table III), followed by processive mult ubiquitination at Lys-48 catalyzed by E220K or CDC34. We are currently testing whether these latter isozymes are responsible for the exclusive E3-dependent mult ubiquitination of substrates during commitment to degradation within the general ATP/ubiquitin-dependent proteolytic pathway. Consistent with this model, the kinetically favored E3-independent chain growth of mult ubiquitin homopolymers from a limited number of directly linked ubiquitin moieties catalyzed by E220K and CDC34 is identical to the pattern of E3-dependent adduct formation within the ATP/ubiquitin-dependent degradative pathway observed for intact cells and cell-free extracts (18).

As noted earlier (3, 7) and in this study, the marked specificity exhibited by the different E2 isozymes in E3-independent conjugation is lost in ligation via the E3-dependent pathway. The reticulocyte and yeast E2 isozymes fall into two broad categories based on the relative affinities for binding of their thiol esters to E3 and their maximal rates of E3-dependent ligation in crude extracts (Table III). This grouping may reflect inherent structural differences in their interaction with a single E3 species or selective interactions with two or more E3 isozymes. The existence of multiple E3 species was originally proposed by Lee et al. (30) and more recently by Ress and Herskho (31). Studies are currently in progress to discriminate between these alternative interpretations. We have recently shown that E220K, E220K, RAD6, and CDC34
support the complete E3-dependent degradative pathway in E2,$\text{E}_{2\text{K}}$-depleted reticulocyte Fraction II.\(^6\)

Demonstration that E2,$\text{E}_{2\text{K}}$ and CDC34 catalyze specific multiubiquitination at Lys-48 provides a model system for elucidating the structural features required for forming this specific linkage among the many potential sites available on ubiquitin. Comparing relative rates of conjugation among the three ubiquitin variants suggests a mechanism for directing such selectivity. For both isozymes, formation of the first multiubiquitin linkage does not occur exclusively at Lys-48 (Figs. 6 and 8), although all subsequent multiubiquitin isopeptide linkages in the growing homopolymer chain occur only at this residue (Fig. 6). Degeneracy in formation of the first ubiquitin bond would not be expected if the specificity for Lys-48 required correct binding to a single conjugated ubiquitin moiety. Therefore, it is more likely that a specificity for Lys-48 required correct binding to a single conjugation lines, respectively.

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