Identification of Rabbit Reticulocyte E2\textsubscript{17K} as a UBC7 Homologue and Functional Characterization of Its Core Domain Loop*

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The structural basis by which ubiquitin (Ub)-conjugating enzymes (E2s) determine substrate specificity remains unclear. We cloned rabbit reticulocyte E2\textsubscript{17K} because unlike the similarly sized class I E2s, E2\textsubscript{14K} and UBC4, it is unable to support ubiquitin-protein ligase (E3)-dependent conjugation to endogenous proteins. RNA analysis revealed that this E2 was expressed in all tissues tested, with higher levels in the testis. Analysis of testis RNA from rats of different ages showed that E2\textsubscript{17K} mRNA was induced from days 15 to 30. The predicted amino acid sequence indicates that E2\textsubscript{17K} is a 19.5-kDa class I E2 but differs from other class I enzymes in possessing an insertion of 13 amino acids distal to the active site cysteine. E2\textsubscript{17K} shows 74% amino acid identity with Saccharomyces cerevisiae UBC7, and therefore, we rename it mammalian UBC7. Yeast UBC7 crystal structure indicates that this insertion forms a loop out of the otherwise conserved folding structure. Sequence analysis of E2s had previously suggested that this loop is a hypervariable region and may play a role in substrate specificity. We created mutant UBC7 lacking the loop (UBC7\textsubscript{Δloop}) and a mutant E2\textsubscript{14K} with an inserted loop (E2\textsubscript{14K+loop}) and characterized their biochemical functions. Ubc7\textsubscript{Δloop} had higher affinity for the E1-Ub thiol ester than native UBC7 and permitted conjugation of Ub to selected proteins in the testis but did not permit the broad spectrum E3-dependent conjugation to endogenous reticulocyte proteins. Surprisingly, E2\textsubscript{14K+loop} was unable to accept Ub from ubiquitin-activating enzyme (E1) but was able to accept NEDD8 from E1. E2\textsubscript{14K+loop} was able to support conjugation of NEDD8 to endogenous reticulocyte proteins but with much lower efficiency than E2\textsubscript{14K}. Thus, the loop can influence interactions of the E2 with charged E1 as well as with E3s or substrates, but the exact nature of these interactions depends on divergent sequences in the remaining conserved core domain.

In eukaryotes, selective degradation of many key cellular proteins is mediated by the ubiquitin/proteasome system reviewed in Refs. 1 and 2. These proteins include gene transcription factors (3, 4), mitotic cyclins (5–7), abnormal/mutated proteins (8), oncoproteins (9), the tumor suppressor protein p53 (10), and cell-surface receptors (11). Through degradation of these proteins, the ubiquitin/proteasome system is implicated in the regulation of diverse processes such as gene transcription, DNA repair (12), cell cycle, cellular stress response, receptor endocytosis (13), and antigen processing (14). How the ubiquitin/proteasome system recognizes a multitude of specific substrates remains to be elucidated and is a question of current interest.

Proteins recognized by this system are targeted to hydrolysis by the 26 S proteasome through the covalent attachment of ubiquitin, a highly conserved 76-amino acid protein. Thus, the ubiquitin-conjugating apparatus plays a key role in selection of substrates. The conjugation of ubiquitin to protein substrates is a multistep process involving three enzymes (15). The first step is catalyzed by ubiquitin-activating enzyme (E1).1 In an ATP-hydrolyzing reaction, a ubiquitin adenylyl intermediate is formed, followed by transfer of the C terminus of the ubiquitin to the thiol group of the active site cysteine residue of the E1 enzyme (16). This activated ubiquitin is then transferred from E1 to a specific cysteine residue of one of several ubiquitin-conjugating enzymes (E2s) (17). Some E2s transfer ubiquitin to ubiquitin-protein ligases (E3s) that bind substrates. This step also involves the formation of a thiol ester linkage between ubiquitin and the ligase (18). Other E2s appear to bind to E3s, and in these situations, the E3s appear to act as docking proteins, which bring together the E2 and the substrate (19). Some E2s can transfer ubiquitin to the substrate directly (20). The E2 and/or E3 enzymes finally catalyze isopeptide bond formation between the C terminus of ubiquitin and the e-amino group of internal lysine residues of target proteins. In most cases, conjugation of ubiquitin to ubiquitin moieties already linked to the protein leads to formation of multi-ubiquitin chains attached to the substrate (2). These multi-ubiquitin chains are subsequently recognized by the 26 S proteasome. Proteins are then probably unfolded and translocated into the central cavity of the proteasome where they are degraded to small peptides (21).

A large body of genetic and biochemical evidence indicates that both E2 and E3 enzymes exist as protein families and together define the substrate specificity of the conjugation system (1, 2). Ubiquitin-conjugating enzymes are a family of closely related proteins. Functionally, these have been best characterized in Saccharomyces cerevisiae, in which 13 E2 genes have been described. Inactivation of many yeast E2 genes produces specific phenotypes indicating that different E2s have different functions. For example, the near identical

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1 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; Ub, ubiquitin; E3, ubiquitin-protein ligases; PAGE, polyacrylamide gel electrophoresis; bp, base pair; AMP-PNP, adenosine 5′- triphosphate.
E2s, UBC4/UBC5 are involved in degradation of abnormal proteins, spolulation, and resistance to stress conditions (8). UBC6 is involved in degradation of the yeast transcriptional repressor MATα2 (22). UBC7 is responsible for resistance to cadmium toxicity (23), degradation of MATα2 (22), and certain proteins localized to the endoplasmic reticulum/nuclear envelope (24). This indicates that E2s are involved in determining substrate specificity either by interacting with specific E3s which in turn bind the substrates or less likely by directly recognizing and ligating ubiquitin to a target protein. E2 protein structures have been intensely studied because they may reveal some of the mechanisms underlying recognition of substrates. All E2s have a core domain of about 150 amino acids that shows at least 25% sequence identity. E2s can be divided into four classes (17). Class I enzymes contain only the conserved catalytic core domain. Class II and III enzymes have either extra C-terminal or extra N-terminal extensions attached to the core domain, respectively. Class IV enzymes have both C-terminal and N-terminal extensions. These extensions to the core domain have been proposed to confer specificity for recognition of substrates or E3s (25) or to provide a localization signal (26). However, divergent sequences within the conserved core domain of E2 proteins may also be highly significant for recognizing substrates.

Haas and co-workers (27) initially identified five major E2s in reticulocyte extracts. E214K supports an apparently wide spectrum conjugation of ubiquitin to endogenous proteins (27) and was subsequently determined to be a class I E2 (28). Interestingly, the other prototype class I E2, the UBC4/5 subfamily, also supports this apparent wide spectrum conjugation of ubiquitin to endogenous proteins (29, 30). E220K, E225K, and E227K were subsequently found to be class II E2s and to conjugate ubiquitin in vitro only to selected substrates (31). However, no apparent substrates have been identified for E217K, and its molecular structure remains unexplored. Although it is relatively similar in size to E214K (which actually has a molecular mass of 17.3 kDa) and therefore may also be a class I enzyme, it is clearly functionally distinct, having no identified substrates. Therefore, we decided to explore the molecular basis of this functional difference by characterizing this E2.

**MATERIALS AND METHODS**

Cloning of cDNA Encoding E217K—E217K was purified from rabbit reticulocyte extracts. E214K supports an apparently wide spectrum conjugation of ubiquitin to endogenous proteins (27) and was subsequently determined to be a class I E2 (28). E217K and its molecular structure remains unexplored. Although it is relatively similar in size to E214K (which actually has a molecular mass of 17.3 kDa) and therefore may also be a class I enzyme, it is clearly functionally distinct, having no identified substrates. Therefore, we decided to explore the molecular basis of this functional difference by characterizing this E2.

**Function of the Loop in UBC7**

A UBC7 construct lacking the loop (PGEDKYGERPEE) was derived by a PCR-based mutagenesis method. To prevent misfolding of ubc7Δloop without the loop, the residues adjacent to the loop were mutated to those in E214K. Two overlapping internal primers, one forward and one reverse, were synthesized and both contain the same deletion of nucleotides encoding amino acid residues 97–109 and same number of nucleotides of nucleotides encoding residue 95 from His to Gly; 96 from Gly to Asn (internal forward primer, 5′ agagagtcgctgctgtaatctcagctg3′; internal reverse primer, 5′ tcagatgagagagagacgctg3′). The internal reverse primer and the external forward primer (5′ agagagtcgctgctgtaatctcagctg3′) were used in one PCR reaction to amplify the 5′ part of the E2. The internal forward primer and the external reverse primer containing a BamHI site (5′ gatctcgagtagccaagacgctg3′) were used in another PCR reaction to amplify the 3′ part of the E2. The purified products of these reactions were then mixed together and amplified using only the external forward and reverse primers to produce the full-length product without the 39 nucleotides encoding the 13 extra amino acids. This product was digested with BamHI and subcloned into the pET-11d vector (Novagen) that had been digested with NcoI, filled in with Klenow polymerase, and then digested with BamHI. The 14 amino acid loop was amplified using the same principle as above except that the two overlapping primers contained the same insertion of nucleotides encoding the 13 amino acids (internal forward primer, 5′ anatattgatgtgagacgctg3′; internal reverse primer, 5′ ctagatgagacgctg3′; external forward primer, 5′ tctgagagagagacgctg3′; external reverse primer, 5′ gatctcgagtagccaagacgctg3′). The full-length product of this reaction was then ligated into the pET-11d vector. The purified plasmids containing UBC7, ubc7Δloop, E214K, and E214KΔloop were transformed individually into Escherichia coli BL21 (DE3) (Novagen). Following induction of expression of T7 polymerase by incubation of the bacteria with isopropyl-β-thiogalactopyranoside for 2 h at 30 °C, the cells were harvested, sonicated, and centrifuged at 10,000 × g. The supernatants containing the individual E2s were used in the assays, or the E2s were purified as described below.

**Immunoblotting of the E217K and E217KΔLoop—Bacterial lysates containing E214K or E214KΔloop were resolved on 15% SDS-PAGE gels and transferred to 0.1-μm nitrocellulose membranes. Membranes were probed with anti-E214K antibody, followed by incubation with secondary antibody, 125I-goat anti-rabbit IgG. The membranes were exposed to X-ray film and to a PhosphorImager screen for quantification. PhosphoImager analysis was carried out using a Fuji imaging plate (Fuji Photo Film Co., Ltd.). Various amounts of the lysates were analyzed to confirm linearity of the quantitation.

**Purification of Recombinant NEDD8—A DNA fragment encoding the mature proteolytically processed form of rat NEDD8 was amplified from rat muscle RNA using the reverse transcriptase-PDR, and oligonucleotide primers derived from the 5′ end of the SEDD8 sequence (6) reported previously in GenBank™. The fragment was subcloned into the bacterial expression vector pET11-d (Novagen) and transformed into E. coli BL21 (DE3). Following induction of expression with 1 μg isopropyl-β-thiogalactopyranoside for 2 h at 30 °C, cells were harvested from 800 ml of culture and rinsed with phosphate-buffered saline, and the cell pellets were frozen at −20 °C. Subsequent manipulations were on ice or at
Comparing with Ub C7, the translation of the sequences yielded identical amino acid sequences suggesting the presence of a blocked N terminus. Therefore, the protein was digested with cyanogen bromide.

Three peptides, two of approximately 6 kDa and one of approximately 5 kDa, were generated. Two peptide sequences were obtained, one of 17 residues and another of 16 residues (Fig. 1). By examining data bases, we found that these two peptides obtained, one of 17 residues and another of 16 residues (Fig. 1). By examining data bases, we found that these two peptides were derived from the database. Function of the Loop in UBC7

The chloramine-T method was used to label bovine ubiquitin with Na[125]I to a specific radioactivity of 6,000 cpm/pmol and to label NEDD8 to a specific radioactivity of 15,000 cpm/pmol. Unincorporated [125]I was removed by passing the reaction products over a Sephadex G-25 column.

Thiol Ester Assay—The relative enzymatic activities of E214K, E214K, UBC7, and ubc7 containing bacterial lysates were quantitated by their abilities to form thiol ester complexes with 125I-NEDD8. The ability of bacterial lysates to support conjugation of ubiquitin or NEDD8 to endogenous proteins was tested as follows. The test fraction derived from endogenous ubiquitin or 100 μM E1 and 5 μM [125]I-ubiquitin or 100 μM [125]I-NEDD8 and the indicated concentrations of E2s. Following incubation for the indicated times at 37°C, the reactions were stopped with Laemmli sample buffer containing 2-mercaptoethanol, the products resolved of free ubiquitin or NEDD8 by SDS-PAGE on 10% acrylamide gels, and detected by autoradiography. When required, proteins were cut out and the incorporated ubiquitin or NEDD8 quantitated by counting in a gamma counter. The rabbit reticulocyte fraction devoid of ubiquitin-conjugating enzymes was prepared by a 30% ammonium sulfate precipitation of fraction II as previously described (27).

Preparation of crude testis cytosol and its fractionation by quaternary amine anion exchange chromatography (Amersham Pharmacia Biotech MonoQ) was as described previously (34).

RESULTS AND DISCUSSION

Cloning of E217K—To determine whether E217K is a class I enzyme, we obtained peptide sequence from the purified protein for the purpose of molecular cloning. Direct sequencing of purified rabbit reticulocyte E217K protein did not yield any sequence suggesting the presence of a blocked N terminus. Therefore, the protein was digested with cyanogen bromide.

Three peptides, two of approximately 6 kDa and one of approximately 5 kDa, were generated. Two peptide sequences were obtained, one of 17 residues and another of 16 residues (Fig. 1). By examining data bases, we found that these two peptides revealed significant sequence similarities with E2s in various species. To obtain the cDNA sequence, degenerate primers were designed encoding a part of each peptide sequence (Fig. 1). These primers were used with rabbit reticulocyte, rat testis, kidney, and muscle cDNAs in PCR reactions. A fragment of 207 bp was amplified from all of the tissue cDNAs. Sequencing of the fragments and translation of the sequences yielded identical amino acid sequences that corresponded to the peptide microsequencing results, confirming that the DNA fragment encodes part of E217K. Because RNA analysis with this cDNA cannot be done in a stable form, we chose the E217K sequence for PCR analysis. Function of the Loop in UBC7

Conjugation Assays—The abilities of native and mutated forms of the E2s to support conjugation of ubiquitin or NEDD8 to endogenous proteins were tested as follows. The test fraction derived from endogenous ubiquitin or 100 μM E1 and 5 μM [125]I-ubiquitin or 100 μM [125]I-NEDD8 and the indicated concentrations of E2s. Following incubation for the indicated times at 37°C, the reactions were stopped with Laemmli sample buffer containing 2-mercaptoethanol, the products resolved of free ubiquitin or NEDD8 by SDS-PAGE on 10% acrylamide gels, and detected by autoradiography. When required, proteins were cut out and the incorporated ubiquitin or NEDD8 quantitated by counting in a gamma counter. The rabbit reticulocyte fraction devoid of ubiquitin-conjugating enzymes was prepared by a 30% ammonium sulfate precipitation of fraction II as previously described (27). Preparation of crude testis cytosol and its fractionation by quaternary amine anion exchange chromatography (Amersham Pharmacia Biotech MonoQ) was as described previously (34).
as probe (see below) showed higher expression of E2$_{17K}$ in rat testis (Fig. 2), a cDNA library derived from this tissue was screened to obtain full-length clones. Five positive clones were found, all possessing an open reading frame encoding a protein of 170 amino acids and a predicted mass of 19.5 kDa. Comparison of the sequence with the E2s in the GenBank$^\text{TM}$ data base showed that this protein has 74% amino acid identity with \textit{S. cerevisiae} UBC7 (Fig. 1). It has the conserved core catalytic domain but lacks N- or C-terminal extensions and is therefore a class I enzyme. The core domain contains the active site cysteine and the conserved "HPN" tripeptide found in all E2s. The deduced amino acid sequence was also found to be identical to human E2g (36), confirming again the conservation of the domain but lacks N- or C-terminal extensions and is therefore a class I enzyme. The core domain contains the active site cysteine and the conserved "HPN" tripeptide found in all E2s.

Comparison of UBC7 with Other E2s—To begin determining which tissues express UBC7, RNA from various rat tissues were analyzed by Northern blotting using the fragment of rat UBC7 cDNA as a probe. One transcript (2.5 kilobase pairs) was subcloned into pGEM-T vector and used to synthesize an antisense$^{32}$P-labeled RNA probe. RNA samples were prepared from the indicated rat tissues. Following hybridization of 10–µg aliquots of the RNA samples with the $^{32}$P-labeled riboprobes and digestion by a RNase A/T mixture, the protected fragments were resolved on a 4% denaturing polyacrylamide gel and detected by autoradiography.

Expression of UBC7 in Various Rat Tissues—To determine which tissues express UBC7, RNA from various rat tissues were analyzed by Northern blotting using the fragment of rat UBC7 cDNA as a probe. One transcript (2.5 kilobase pairs) was observed in all rat tissues examined. Thus, this E2 appears to have a general cellular function. This would be consistent with results from studies in yeast which indicate that UBC7 plays a role in the degradation of abnormal proteins in the endoplasmic reticulum (24). When normalized to 18 S rRNA levels, all tissues showed low expression levels, but slightly higher expression was seen in testis (Fig. 2A). This is in contrast to the expression pattern of human E2g which when normalized for actin expression showed similar levels in all tissues (36). This is likely due to our use of tissues from young peripubertal rats for this analysis, whereas the samples of human mRNA in the E2g study were derived from an adult which in the rat has lower testis expression (see below). The more sensitive RNase protection assay confirmed that rat UBC7 mRNA is expressed diffusely with a slightly higher expression in testis (Fig. 2B).

Expression of UBC7 in Testis—To begin determining which tissues express UBC7, RNA from various rat tissues were analyzed by Northern blotting using the fragment of rat UBC7 cDNA as a probe. One transcript (2.5 kilobase pairs) was subcloned into pGEM-T vector and used to synthesize an antisense$^{32}$P-labeled RNA probe. RNA samples were prepared from the indicated rat tissues. Following hybridization of 10–µg aliquots of the RNA samples with the $^{32}$P-labeled riboprobes and digestion by a RNase A/T mixture, the protected fragments were resolved on a 4% denaturing polyacrylamide gel and detected by autoradiography.

Expression of UBC7 in Various Rat Tissues—To determine which tissues express UBC7, RNA from various rat tissues were analyzed by Northern blotting using the fragment of rat UBC7 cDNA as a probe. One transcript (2.5 kilobase pairs) was observed in all rat tissues examined. Thus, this E2 appears to have a general cellular function. This would be consistent with results from studies in yeast which indicate that UBC7 plays a role in the degradation of abnormal proteins in the endoplasmic reticulum (24). When normalized to 18 S rRNA levels, all tissues showed low expression levels, but slightly higher expression was seen in testis (Fig. 2A). This is in contrast to the expression pattern of human E2g which when normalized for actin expression showed similar levels in all tissues (36). This is likely due to our use of tissues from young peripubertal rats for this analysis, whereas the samples of human mRNA in the E2g study were derived from an adult which in the rat has lower testis expression (see below). The more sensitive RNase protection assay confirmed that rat UBC7 mRNA is expressed diffusely with a slightly higher expression in testis (Fig. 2B).

Since the testis undergoes major postnatal development, we tested whether rat UBC7 expression was developmentally regulated. RNAs from testes of rats of different ages were analyzed by Northern blotting for the expression of UBC7 with the 207-bp fragment as a probe. UBC7 expression was low in testes from 10-day-old rats, increased in testes from 15- to 30-day-old rats, and then decreased upon further maturation to the adult testis (Fig. 3). The peak expression during days 15–30 of life suggests that UBC7 mRNA is present in spermatocytes and possibly early spermatids which make their first appearance in the testis during this period. The subsequent fall in expression in older animals suggests that the mRNA is less prominent or absent in more mature spermatids which begin to accumulate in the testis after 30 days of age. We have previously observed regulation of expression of other E2s during development of the testis, particularly the UBC4 isoforms. UBC4-testis is induced in spermatids (34); UBC4-1 is present in all cells but with higher expression in spermatocytes, and UBC4-2 shows maximal expression in elongated spermatids. Thus, these E2s are likely induced at different stages to target-specific populations of proteins for degradation during this complex developmental process.

Comparison of UBC7 with Other E2s—To begin determining why UBC7, a class I enzyme, is functionally distinct from other

\cite{Rajapurohitam, Morales, El-Alfy, Lefranc, Bedard, Wing, S. S. (1999) Dev. Biol., in press.}
Function of the Loop in UBC7

Characterization of Rat UBC7 and ubc7Δloop—To address this question, the 13 extra residues were deleted from UBC7 to form ubc7Δloop. Native rat UBC7 and ubc7Δloop were expressed in E. coli. Thiols ester assays were performed to test if native UBC7 and ubc7Δloop can accept ubiquitin from E1. In the presence of 125I-labeled ubiquitin and purified E1, both UBC7 and ubc7Δloop can form thiol esters with 125I-ubiquitin (Fig. 4A). However, these end point assays are unable to detect more subtle differences in interaction with E1. Therefore, we measured apparent Km values of UBC7 and ubc7Δloop for the E1-ubiquitin thiol ester (Table I). Interestingly, removal of the loop decreased the Km value while maintaining similar Vmax values indicating that the loop can negatively influence binding of the E2 to the charged E1.

We next tested whether UBC7 and ubc7Δloop can support E3-mediated ubiquitination to endogenous proteins of the reticulocyte extract. As was shown previously, E214K and UBC4 can efficiently support incorporation of ubiquitin into proteins, whereas native UBC7 does not (27). To confirm that this inability of UBC7 was not due to the higher apparent Km for charged E1 than E214K (31), we repeated the assay using a 10-fold higher concentration of UBC7. Even such high levels were unable to support conjugation to any endogenous proteins (data not shown). Removal of the loop in UBC7 did not render it capable of supporting conjugation of ubiquitin to a broad spectrum of endogenous substrates (Fig. 4B). Thus, divergent sequences in the conserved core domain itself must play important roles in determining specificity. Indeed, a few as four amino acid substitutions scattered across a large surface of the E2 core domain has previously been shown to alter specificity of conjugation (40). Interestingly, although broad spectrum incorporation of ubiquitin to protein was not observed, one specific ubiquitinated band was detected in the assays with ubc7Δloop. Thus, the loop may still play a role in specifying substrate selectivity (Fig. 4B).

Since UBC7 appeared more highly expressed in the testis, we tried to identify specific substrates in this tissue. We fractionated the extracts of testes from rats of different ages by anion exchange chromatography, and we screened the fractions for the ability to support conjugation of ubiquitin to proteins in the presence of E1 and either UBC7 or ubc7Δloop. Despite screening fractions derived from cytosolic, nuclear, and membrane preparations of the testis, no fractions could be identified that stimulated conjugation of ubiquitin to proteins in a UBC7-dependent manner (data not shown). Screening fractions in these assays in the presence of ubiquitin aldehyde, an inhibitor of many isopeptidases, also did not reveal any substrates (data not shown). These assays assume either that the E2 can directly conjugate to a protein substrate in the fraction or that the fraction contains both a UBC7-dependent E3 and the substrate. Since it is possible that the E3 and substrate may become separated during fractionation, pools of the fractions were made and assayed together in various combinations. Since S. cerevisiae UBC7 is known to support endoplasmic reticulum-associated degradation through binding to Cue1p (41), an integral endoplasmic reticulum membrane protein, we
also mixed fractions from solubilized testis membranes in these assays. However, there was still no detectable conjugation supported by UBC7 (data not shown). Thus, UBC7 would appear to have very specific substrates in the cell which may be present at low levels and not detectable by our assay. Although genetic studies have indicated that S. cerevisiae UBC7 is involved in the degradation of abnormal proteins in the endoplasmic reticulum (24), it is quite likely that under normal conditions the number of substrates using this degradative system is limited. To date, the only known physiological substrate of S. cerevisiae UBC7 is the MATα2 repressor protein in yeast (22). In contrast to the absence of detectable UBC7 substrates, when ubc7Δloop was used in these assays, incorporation of 125I-ubiquitin into proteins was observed in fractions eluting at approximately 0.35 M NaCl following MonoQ chromatography of the cytosolic fraction (Fig. 4C). Thus, although the loop is not responsible for the inability of UBC7 to support broad spectrum conjugation to endogenous proteins, it can play a role in more subtle substrate selectivity.

Characterization of E214K and E214KΔLoop—To evaluate more carefully the role of the loop, we inserted the 13 extra residues into E214K to form E214KΔloop, and we determined the effects of this insertion. To test if E214KΔloop can accept ubiquitin from E1, thiol ester assays were performed in vitro. In the presence of 125I-ubiquitin and purified E1, bacterial lysate expressing wild type E214K could form a thiol ester complex with 125I-ubiquitin, but lysates expressing E214KΔloop could not (Fig. 5A). This was not due to lack of expression because Western blot analysis showed that the E214KΔloop was actually expressed at twice the level of E214K. The inability to accept ubiquitin from E1 raised the possibility that E214KΔloop did not fold properly. Recently, we found that E1 can activate the ubiquitin homologue NEDD8 which is 60% amino acid identical and 80% similar to ubiquitin, but binds it more weakly than ubiquitin (apparent \(K_m\) NEDD8~25 \(\mu\)M). However, we found that both E214K and E214KΔloop could form thiol ester complexes with NEDD8 in the presence of 125I-NEDD8 and purified E1 (Fig. 5A). Thus the inability of the E214KΔloop to accept 125I-ubiquitin was not due to a major folding defect and UBC7 that like the loop can affect interaction of the E2 with E1 charged with ubiquitin.

Since the loop did have some effects on the ability of UBC7 to recognize substrate, we tested whether the presence of the loop affected the ability of E214K to support conjugation to substrates. Since E214KΔloop could form a thiol ester with NEDD8, we tested whether it can support NEDD8 conjugation to endogenous proteins in the rabbit reticulocyte extract. Na-

### Table I

**Removal of the loop in UBC7 enhances binding to the E1-Ub thiol ester**

Various concentrations of UBC7 or ubc7Δloop were incubated with limiting concentrations of E1 in the presence of 125I-ubiquitin, ATP, and inorganic pyrophosphatase. The initial velocities of transfer of the 125I-ubiquitin from E1 to the E2 were determined at various concentrations of E2 and used in double-reciprocal plots to determine the apparent \(K_m\) and \(V_{max}\) values. Shown are the means ± S.E. for three independent measurements.

|        | \(K_m\) \(\mu\)M | \(V_{max}\) fmol Ub/min |
|--------|-------------------|----------------------------|
| UBC7   | 283 ± 9           | 299 ± 9                    |
| ubc7Δloop | 127 ± 23         | 263 ± 43                   |

\(a\) Values for UBC7 and ubc7Δloop significantly different (\(p < 0.001\)).

\(b\) Values for UBC7 and ubc7Δloop not significantly different (\(p > 0.35\)).

3 S. S. Wing, unpublished data.

![Fig. 5. Characterization of E217K and E217KΔloop](image)
of the E2 to accept ubiquitin from E1. This latter feature could play a role in regulating substrate conjugation by rendering more favorable the interaction of some E2s with E1 charged with ubiquitin or possibly other ubiquitin-like molecules. More importantly, though, the effects of the loop on substrate conjugation and on interaction with E1 differed in UBC7 and E2\textsubscript{14K}, indicating that the precise effects were dependent on the nature of the core domain where the loop was attached. Thus, as was shown for the C-terminal extension of E2\textsubscript{225K}, the function of this additional segment is dependent on sequences in the core (42). Previous studies showing that transfer of the C-terminal extension of CDC34 onto the core domain of RAD6 can confer CDC34 functions to the chimeric molecule (25, 43) would suggest that specificity of conjugation can be ascribed to these additional sequences and that catalytic functions can be ascribed to the core domain. However, our findings propose a more complex model in which specificity of function is determined together by both variable sequences in the core and the additional sequence elements.

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