Identification of Amino Acid Residues in a Class I Ubiquitin-conjugating Enzyme Involved in Determining Specificity of Conjugation of Ubiquitin to Proteins*

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The ubiquitin pathway is a major system for selective proteolysis in eukaryotes. However, the mechanisms underlying substrate selectivity by the ubiquitin system remain unclear. We previously identified isoforms of a rat ubiquitin-conjugating enzyme (E2) homologous to the Saccharomyces cerevisiae class I E2 genes, UBC4/UBC5. Two isoforms, although 90% identical, show distinct features. UBC4-1 is expressed ubiquitously, whereas UBC4-testis is expressed in spermatids. Interestingly, although these isoforms interacted similarly with some ubiquitin-protein ligases (E3s) such as E6-AP and rat p100 and an E3 that conjugates ubiquitin to histone H2A, they also supported conjugation of ubiquitin to distinct subsets of testis proteins. UBC4-1 showed an 11-fold greater ability to support conjugation of ubiquitin to endogenous substrates present in a testis nuclear fraction. Site-directed mutagenesis of the UBC4-testis isoform was undertaken to identify regions of the molecule responsible for the observed difference in substrate specificity. Four residues (Gln-15, Ala-49, Ser-107, and Gln-125) scattered on surfaces away from the active site appeared necessary and sufficient for UBC4-1-like conjugation. These four residues identify a large surface of the E2 core domain that may represent an area of the substrate (14). E3s are important for recognition and binding of the substrate (14). E3s may serve as docking proteins that substrates are selected by the ubiquitin-conjugating apparatus remain unclear. E3s may function as the final intermediate in the ubiquitin thiol ester cascade (17). The E3 E6-AP (E6-associated protein) forms a thiol ester linkage with ubiquitin prior to catalyzing the ubiquitination of p53 in the presence of the viral E6 protein (17). The catalytically active cysteine in E6-AP is found within its carboxyl terminus domain, and a number of putative E3s have been identified based on the presence of such HECT (homology to E6-AP carboxyl terminus) domains (18). Although E3s bind substrates, E2s may also be involved in substrate recognition either by conjugating substrates directly or probably more commonly by interacting only with specific E3s. Indeed, ubiquitin-mediated proteolysis is responsible for the turnover of key regulatory proteins, including mitotic cyclins (cyclin B) (4, 5), cyclin-dependent kinases (Sic1 and p27) (6, 7), and transcription factors (Mato2, c-Jun, and p53) (8–10).

Recognition of specific substrates occurs at the level of conjugation, which is a multistep process involving three types of enzymes (11): a ubiquitin-activating enzyme (E1), one ubiquitin-conjugating enzymes (UBCs or E2s), and, in many cases, ubiquitin-protein ligases (E3s). Initially, ubiquitin is activated by E1 through the ATP-dependent formation of a thiol ester bond between ubiquitin and E1 (12). The activated ubiquitin is then transferred via a thiol ester linkage to a cysteine residue of an E2 (reviewed in Ref. 13). Finally, the E2 itself, or more commonly in concert with an E3, ligates the ubiquitin via its carboxyl terminus to lysine residues of a protein substrate. Successive ubiquitin molecules may be added to lysine residues of the previous ubiquitin to produce a multi-ubiquitin chain. Although the biochemical mechanisms of the pathway are becoming well defined, the molecular mechanisms by which substrates are selected by the ubiquitin-conjugating apparatus remain unclear. E3s are important for recognition and binding of the substrate (14). E3s may serve as docking proteins that bind both specific substrates and E2s (14), thereby permitting the transfer of ubiquitin from an E2 to a substrate. For example, the E3 SCP3/4 binds the E2 molecule Cdc4 and a specific substrate, Sic1, simultaneously, thereby facilitating the transfer of ubiquitin from Cdc4 to Sic1, an inhibitor of the yeast S-phase cyclin-dependent kinase Cln1-Cdc28 (15, 16). Alternatively, E3s may function as the final intermediate in the ubiquitin thiol ester cascade (17). The E3 E6-AP (E6-associated protein) forms a thiol ester linkage with ubiquitin prior to catalyzing the ubiquitination of p53 in the presence of the viral E6 protein (17). The catalytically active cysteine in E6-AP is found within its carboxyl terminus domain, and a number of putative E3s have been identified based on the presence of such HECT (homology to E6-AP carboxyl terminus) domains (18).

Although E3s bind substrates, E2s may also be involved in substrate recognition either by conjugating substrates directly or probably more commonly by interacting only with specific E3s. Indeed, yeast genetic studies have revealed a variety of functions for different E2s indicating that they can direct conjugation of ubiquitin to specific substrates. For example, UBC2 (RAD6) is required for DNA repair (19), whereas UBC4/UBC5

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†. The abbreviations used are: E1, ubiquitin-activating enzyme; E2 and UBC, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; PCR, polymerase chain reaction; GST, glutathione S-transferase; DTT, dithiothreitol; AMP-PNP, 5′-adenyl imidodiphosphate; RM-Ub, reductively methylated ubiquitin.
are required for the degradation of short-lived and abnormal proteins (20).

Differences in E2 function evidently reflect differences in E2 structure. E2 enzymes have been divided into four structural classes based on amino acid sequence comparison (21). Class I enzymes (e.g. Ubc4 and Ubc5) (20) consist of a conserved catalytic core domain of ~150 amino acids that contains the active-site cysteine involved in ubiquitin transfer. Class II enzymes (e.g. Ubc2/Rad6 and Ubc3/Cdc34) (19, 22) have extra C-terminal extensions or tails attached to the core domain, whereas class III enzymes (e.g. Ubc7 are also depicted. The active-site cysteine is indicated by an arrowhead. The critical residues that confer substrate specificity. More recently, the C-terminal tail of E2–25K must possess determinants of function and by inference substrate specificity. The tail is required for DNA repair, as well as for functions, indicating that the tail depends on structural features that are critical for substrate specificity. Two isoforms of rat UBC4, although 93% identical, show distinct features. Rat UBC4-testis possesses an acidic pI and is expressed ubiquitously (29). Therefore, although the high degree of sequence similarity might suggest that these isoforms are redundant, the highly regulated and cell-specific expression suggested a unique role for the UBC4-testis isoform.

We also determined whether these two isoforms interact differently with other E3s. In addition to interactions described (29, 30), we have also been able to confirm these interactions in vitro with different substrates present in a testis nuclear fraction (30). We also determined whether these two isoforms interact differentially with other E3s. In addition to interactions described (29, 30), we have also been able to confirm these interactions in vitro with different substrates present in a testis nuclear fraction (30).

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis—PET-11d (Novagen)-based Escherichia coli expression plasmids encoding rat UBC4-1 or UBC4-testis have been described (29, 30). Mutagenesis of selected residues in UBC4-testis to those in UBC4-1 was performed using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Briefly, separate mutagenic primers encoding site-specific mutations in UBC4-testis and a selection primer were annealed to denatured UBC4-testis-containing pET-11d plasmids, and a reaction mixture was transformed into Escherichia coli TG1. Transformants were selected on imipenem plates and verified by DNA sequence analysis. Site-directed Mutagenesis—


type II enzymes (e.g. Ubc6/Ubc5) (29, 30). Therefore, we characterized carefully the abilities of rat UBC4-1 and UBC4-testis to support conjugation of ubiquitin in vitro to different subsets of testis proteins. Rat UBC4-1 shows an 11-fold greater ability to support conjugation of ubiquitin to endogenous substrates than does rat UBC4-1, which has a basic pI and is expressed ubiquitously (29). Therefore, although the high degree of sequence similarity might suggest that these isoforms are redundant, the highly regulated and cell-specific expression suggested a unique role for the UBC4-testis isoform.

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2 Previously, the rat homologue of yeast Ubc4 was referred to as E2–YBR and included isoforms 2E and 8A (29, 30). For purposes of clarity and to conform to a trend by workers in the field to name E2s after their apparent yeast homologs, isoform 2E will henceforth be referred to as rat UBC4-1, and isoform 8A as rat UBC4-testis. The nucleotide sequences of UBC4-1 and UBC4-testis have been submitted to GenBank™ with accession numbers U13177 and U56407.

3 Oligonucleotide sequences and detailed PCR conditions are available on request.
and the mutant DNA strand was extended with T7 DNA polymerase and ligated with T4 DNA ligase. The selection primer, located ~2 kilobases from the mutagenic primers, changed the unique AciI restriction site on pET-11d to a unique KpnI restriction site on the mutant plasmid strand, thereby permitting selection of intact mutant plasmids by AciI/ KpnI digestion. Initially, the desired fragments were transfected into a D55H, E68A, or double D55H/E68A substitution were generated. Similarly, subsequent mutations were added separately or in combination onto the initial UBC4-testis D55H/E68A double mutant in the following order: Gln-15, Gln-125, Ala-49, or Ser-107. The intact mutant plasmids were transformed into E. coli XL1-Blue cells (Stratagene). All plasmids were sequenced to confirm the desired mutation using the fmol™ DNA sequencing System (Promega). Subtractive mutagenesis was then performed in a similar manner to define the minimal UBC4-1 residues on UBC4-testis that were necessary and sufficient for the UBC4-1-like conjugating activity.

The converse mutagenesis of the four critical residues (Arg-15, Val-49, Cys-107, and Arg-125) of UBC4-1 to those of UBC4-testis was performed via PCR amplification (32). Mutant UBC4-1 fragments were generated using UBC4-1-containing pET-11d as a template, primers bearing the relevant base substitutions, and sense or antisense oligonucleotides encoding the amino and carboxyl termini of the protein as well as restriction sites to permit cloning into the NcoI and BamHI sites of pET-11d. The mutant UBC4-1 fragments were then purified by agarose gel electrophoresis and incorporated into full-length mutant UBC4-1 by a second round of PCR amplification using separate mutant UBC4-1 fragments as a template and both the 5'-NcoI and the 3'-BamHI primers. The PCR products were purified on a QiAquick PCR purification column (QIAGEN Inc.), digested with NcoI and BamHI, and then ligated into a pET-11d vector that had been digested with the same enzymes. Purified plasmids were transformed into XL1-Blue cells, and individual positive clones were sequenced using the fmol™ DNA sequencing system to confirm the presence of the desired mutation.

Preparation of Proteins—The pGEX-ubiquitin plasmid encoding the GST-ubiquitin fusion protein (33) was expressed in E. coli strain DH5α and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 37 °C. Bacterial cell pellets were resuspended in phosphate-buffered saline and 1% Triton X-100 were lysed by sonication and clarified by centrifugation at 12,000 × g. Glutathione-Sepharose (Amersham Pharmacia Biotech) was added to the supernatant, and the mixture was rotated overnight at 4 °C. Beads were washed in phosphate-buffered saline and 1% Triton X-100 and eluted in 20 mM glutathione in phosphate-buffered saline for 20 min at 25 °C. The GST-ubiquitin fusion protein content of the beads was analyzed by Coomassie Blue staining.

E1 was prepared from rabbit liver. Bacterially expressed recombinant UBC4-1 and UBC4-testis proteins were also purified as described previously (29, 30). The E1, UBC4-1, and UBC4-testis enzymes were purified by quantitative initial release of radioactive pyrophosphate following incubation in the presence of γ-32PATP and ubiquitin (34).

The purified pET-11d-based plasmids containing the mutant UBC4-1 and UBC4-testis genes were transformed into E. coli BL21 (DE3) (Novagen), and induction of the recombinant proteins with 1 mM isopropyl-β-D-thiogalactopyranoside was carried out for 2 h at 30 °C. The cells were pelleted and resuspended in 0.1 volume and then lysed by sonication in 50 mM Tris, pH 7.5, and 1 mM DTT. Cellular debris was removed by centrifugation at 12,000 × g. The enzymatic activities of the mutant E2-containing bacterial lysates relative to those of the purified native E2s were measured using the fmol™ DNA sequencing system to confirm the presence of the desired mutation.

**Thiol Ester Assays—**To determine the minimal UBC4-1 residues on UBC4-testis that were necessary and sufficient for the UBC4-1-like conjugating activity.

The [35S]methionine-labeled E2s E6-AP (35) and rat p100 (36), covalently bound to GST-ubiquitin fusion protein in thiol ester linkages (17, 18), were detected by incubating the enzymes in the presence of 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM MgCl2, 2 mM ATP, 50 mM E1, 20 units/ml inorganic pyrophosphatase, 500 nM E2, and 2 μl of each partially purified and concentrated E2 preparation. The reaction mixtures were then incubated at 25 °C for 3 min, and then the reaction was initiated with 1 μg of GST-ubiquitin fusion protein (33), incubated at 25 °C for 5 min, and stopped with Laemmli sample buffer with or without β-mercaptoethanol. The reactions were resolved at 4 °C on an SDS-12.5% polyacrylamide gel, which was then soaked in ENHANCE (NEW Life Science Products) and autoradiographed.

**Conjugation Assays—**For the conjugation of ubiquitin to endogenous substrates present in the testis nuclear fraction, the reaction mixture contained the following in a final volume of 20 μl: 10 μl of the 0.05 mM NaCl nuclear fraction, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM MgCl2, 2 mM AMP-PNP, 5 μM 125I-ubiquitin (3000 cpm/μmol), 50 mM E1, and 250 nM E2s. The ubiquitination rate for the nuclear fraction was linear for 1 h at 37 °C, and this assay was performed in the presence or absence of 0.5 μg of ubiquitin or 40 μM MG132 (Proscript), an isopeptidase inhibitor, or 40 μM MG132 (Proscript), a proteasome inhibitor.

For the conjugation of ubiquitin to the exogenous substrate histone H2A, mediated by the testis cytosolic E3, the reaction mixture contained the following in a final volume of 20 μl: 5 μl of the cytosolic E3 Superdex 200 fraction, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM MgCl2, 2 mM ATP, 0.5 units pyrophosphatase, 12.5 mM phosphocreatine, 2.5 units of creatine kinase, 250 mM E1, 125I-histone H2A (specific activity of 375,000 cpm/μg), and varied concentrations of E2s as indicated. Reactions were initiated with 25 μM reductively methylated ubiquitin (RM-Ub), prepared, and quantitated as described (38). Since histone H2A contains a number of lysine residues, mono- to penta-RM-Ub conjugates were formed, and the rate of formation of these conjugates was found to be linear for 10 min at 30 °C, permitting their quantification.

**RESULTS**

**Differential Abilities of Rat UBC4-1 and UBC4-testis to Conjugate Ubiquitin to a Fraction of Testis Nuclear Proteins—**To test whether the structural differences between rat UBC4-1 and UBC4-testis conferred different abilities to conjugate ubiquitin to proteins, ubiquitination assays were performed using testis extracts fractionated on a MonoQ anion-exchange column. As shown previously (30), a nuclear fraction eluting at 0.05 M NaCl supported conjugation of ubiquitin to proteins essentially only with the UBC4-1 isoform (Fig. 2A). This indicated that these two isoforms showed differential substrate specificity and suggested an enhanced ability of the ubiquitous isoform to conjugate ubiquitin to endogenous proteins in this fraction. To evaluate the possibility that UBC4-testis was conjugating ubiquitin to proteins that are preferentially de-ubiq-
ubiquitinated by a co-purifying isopeptidase activity, the conjugation assay was performed in the presence of the isopeptidase inhibitor ubiquitin aldehyde. Notably, the level of UBC4-testis-dependent conjugation was not increased by the addition of this reagent, rendering unlikely the possibility of a co-purifying interfering isopeptidase. Likewise, to rule out the possibility of enhanced proteasomal degradation of the UBC4-testis-dependent conjugates, the conjugation assay was performed in the presence of the proteasomal inhibitor MG132. Similarly, MG132 did not increase the levels of UBC4-testis-dependent conjugates.

Significantly, the observed difference in conjugating ability between UBC4-1 and UBC4-testis was not due to a difference in the ability of these E2s to accept ubiquitin from E1 because UBC4-1 and UBC4-testis formed similar amounts of ubiquitin thiol esters (Fig. 2B). These thiol ester assays are end-point assays, and the results cannot exclude the possibility of different affinities of these two isoforms for E1. However, more detailed thiol ester-based enzyme kinetic studies suggest that UBC4-1 and UBC4-testis show less than a 2-fold difference in their affinities for E1 (data not shown).

**Rat Isoforms UBC4-1 and UBC4-testis Interact with the E3s E6-AP and rat p100.** Since the observed difference in conjugation by rat UBC4-1 and UBC4-testis was found not to be due to significant differences in their interaction with E1, this suggested that the specificity might arise at the E2-E3 level. We therefore tested the abilities of rat UBC4-1 and UBC4-testis to interact with some well defined E3s, E6-AP (10, 17) and rat p100 (18), which contain HECT domains and thereby form thiol ester linkages with ubiquitin by accepting ubiquitin from E2 thiol esters. We tested the abilities of rat UBC4-1 and UBC4-testis to transfer ubiquitin to E6-AP and rat p100 produced by in vitro translation (Fig. 3). Since these E3s are relatively large, a GST-ubiquitin fusion protein (molecular mass of 34 kDa) (18) was used to permit resolution of the E3-ubiquitin thiol ester linkage by gel electrophoresis. In the presence of E1, GST-ubiquitin, and rat UBC4-1 or UBC4-testis, bands ~34 kDa larger than the expected translation products were evident. These bands were not present when the thiol ester reactions were treated with β-mercaptoethanol or when the reaction was performed in the absence of rat UBC4-1 and UBC4-testis. Again, these results obtained with thiol ester assays do not eliminate the possibility that these E2 isoforms could differ somewhat in their affinities for these E3s. More detailed kinetic assays are not feasible in these crude preparations from in vitro translation, where the concentrations of the E3s cannot be readily determined. Nonetheless, the results demonstrated that these two isoforms are both capable of interacting with ubiquitin-protein ligases as shown by their ability to transfer ubiquitin to at least two different E3s, E6-AP and rat p100.

**Rat Isoforms UBC4-1 and UBC4-testis Support Ubiquitination of Histone H2A Mediated by a Testis Cytosolic E3.** Since the assays described above do not permit ready quantitative determinations of reaction rates, we tested both isoforms for their ability to support conjugation of ubiquitin to an exogenous substrate, histone H2A, mediated by a testis E3. To this end, a testis cytosolic E3 activity (29, 30) eluting at 0.4 M NaCl from a MonoQ anion-exchange column was further fractionated using a gel filtration column. This E3 activity was found to support conjugation of ubiquitin to the exogenous substrate 125I-labeled histone H2A in the presence of rat UBC4-1 and UBC4-testis. The effectiveness of these two isoforms in supporting this E3-dependent ubiquitination of 125I-histone H2A was compared using E1, an ATP-regenerating system, RM-Ub (38), and different concentrations of rat UBC4-1 and UBC4-testis (Fig. 4A). RM-Ub was utilized in the assays to restrict the ubiquitination of histone H2A to the mono-ubiquitinated form, which would facilitate the quantification of conjugates. Multi-ubiquitinated forms of histone H2A were observed, indicating that ubiquitin is attached to several different lysine residues of the molecule. In contrast to the differential ubiquitin-conjugating abilities of rat UBC4-1 and UBC4-testis observed in the
E2 Residues Determining Substrate Specificity

Four Amino Acid Differences Are Responsible for the Abilities of Rat Isoforms UBC4-1 and UBC4-testis to Conjugate Ubiquitin to Different Subsets of Testis Nuclear Proteins—Since the rat UBC4-1 and UBC4-testis isoforms differ by only 11 amino acids (Fig. 1) (30), this provided an unprecedented opportunity to identify, by site-directed mutagenesis, critical residues of the E2 molecule responsible for the observed difference in substrate specificity. Site-directed mutagenesis of the UBC4-testis isoform was undertaken to identify regions of the molecule that can confer the UBC4-1-like ability to conjugate ubiquitin to the endogenous substrates present in the nuclear fraction.

Since rat UBC4-1 and UBC4-testis differ with respect to their pI values (30), mutagenesis of UBC4-testis began with the four residues responsible for the dramatic change in pI. Of particular interest were the two residues, aspartic acid 55 and glutamic acid 68, located near the active-site cysteine, that were mutated to histidine and alanine, respectively. However, mutation of UBC4-testis to the UBC4-1 residues at these two sites did not confer UBC4-1-like ability to conjugate 125I-ubiquitin to the substrates present in the testis nuclear fraction (Fig. 5A). Additional mutagenesis of glutamines 15 and 125, the two other residues responsible for the change in pI, to arginines resulted in a small increase in conjugating activity of the mutated UBC4-testis. Since mutagenesis of the two glutamine residues appeared to increase the conjugating activity of the mutated UBC4-testis, further residues nearby were mutated. Mutation of alanine 49 to valine conferred some increase in activity, and an additional mutation of serine 107 to cysteine conferred a UBC4-1 phenotype in conjugating ability to the mutated UBC4-testis.

To determine the minimal number of substitutions required for the UBC4-1-like conjugating activity of the mutated UBC4-testis, subtractive mutagenesis was then performed. Since mutagenesis of Gln-15, Ala-49, Ser-107, and Gln-125 improved conjugating activity, these four mutations were tested together and found to be sufficient (Fig. 5A). Further removal of any one of these substitutions decreased conjugating ability significantly, and therefore, these four substitutions are necessary (Fig. 5B).

Our observation that these residues are important would predict that mutating the rat UBC4-1 molecule at these positions to the UBC4-testis residues would decrease the conjugating activity of UBC4-1. To test this prediction, mutagenesis of UBC4-1 to UBC4-testis was performed at these four residues. As expected, mutagenesis of each of the critical residues (Arg-15, Val-49, Cys-107, or Arg-125) resulted in decreased conjugating activity of rat UBC4-1 in the testis nuclear fraction (Fig. 6, A and B). Interestingly, the four residues in UBC4-testis, which were found to be necessary and sufficient for the UBC4-1-like conjugating activity, are present on surfaces away from the active site (Fig. 7) (31).

DISCUSSION

We have, for the first time, identified, in the core domain conserved among all ubiquitin-conjugating enzymes, specific sites that are involved in determining substrate specificity of conjugation. These studies revealed a number of intriguing insights. First, although we anticipated, based on the 93% amino acid identity between the two isoforms, that only a limited number of residues in the core domain of E2s may dictate conjugation of ubiquitin to different proteins, surpris-
ingly only four substitutions (Figs. 5 and 6) were sufficient and necessary to produce the change in substrate specificity.

Second, some of these substitutions appeared to decrease conjugation ability, and therefore, these four substitutions appear necessary. C, the conjugating activities of the UBC4-testis mutants relative to the UBC4-1 isoform were compared by counting the radioactivity in the gel lanes (±S.E.) and were normalized to the value for UBC4-1. D, Asp-55; E, Glu-68; Q1, Gln-15; Q2, Gln-125; A, Ala-49; S, Ser-107.

Although all E2s exhibit limited sequence identity and are functionally different, the overall three-dimensional folding of E2 core domains that have been crystallized to date is remarkably similar (31, 40–42). The 150 amino acids of the E2 core domains show 25% sequence identity, and notably, most of the identical residues are either buried or clustered on one surface adjacent to the active-site cysteine (31). It has been suggested that the highly conserved surface region around the active site may be specific for ubiquitin and/or E1 binding, whereas the divergent surface regions may enable individual E2 enzymes to bind their respective substrates or E3s (31). Our data now provide experimental evidence to support this hypothesis.

Although we have not to date been able to identify an exogenous substrate that requires the nuclear fraction for conjugation, other studies (30, 43) showing that this family of E2s interacts extensively with E3s would suggest that the nuclear fraction probably does contain an E3 activity. Recent findings suggest that a putative E2-binding site exists in the C terminus of HECT domain-containing E3s and that a variable E3 N terminus may be involved in binding substrates (44). Since the residues found to be critical for the UBC4-1-like conjugating activity lie on a surface away from the active site, this surface may be responsible for the selective interaction of UBC4-1 with a nuclear E3. This could permit the small E2 molecule, while bound to the larger E3 protein (known E3s are >95 kDa in size), to expose its active-site cysteine, thereby facilitating the transfer of ubiquitin to the E3 if it contains a HECT domain or directly to a substrate if the E3 is functioning primarily as a docking protein (Fig. 8).

Fourth, it is possible that some E2s can functionally overlap with some E3s or substrates, yet be selective for other E3s or
substrates. For example, the critical residues that confer a UBC4-1-like phenotype on UBC4-testis likely result in a distinctive E2 surface configuration that is selectively recognized by a specific nuclear E3. However, other E3s, such as rat p100, E6-AP, and the testis cytosolic E3 we have identified, may interact principally with residues that are conserved among these E2s (Figs. 3 and 4). Indeed, the functional specificity of distinct E2s may be contingent upon specific residues in these molecules that facilitate or impair their interaction with different E3s or substrates. In support of this, it has been shown that the mouse homologues of yeast Rad6, mHR6A and mHR6B, which show 95% amino acid sequence identity (Fig. 1), may be functionally distinct since inactivation of the mHR6A gene, but not the mHR6B gene, in mice causes male sterility (45). Thus, mHR6A appears to complement some functions of mHR6B, but not all of them. The minor sequence differences between the two isoforms may therefore be responsible for the selectivity of binding to some E3s or substrates.

Recently, three-dimensional structure determination of yeast Ubc7 (41) revealed that its tertiary folding is similar to other class I enzymes that have been crystallized, with the exception of two regions where extra residues are present in Ubc7. Based on amino acid sequence alignment between 13 yeast E2 enzymes, Cook et al. (41) suggested that there are four potential regions where extra residues could be inserted into the common core domain of various E2s and that these may represent hypervariable regions that confer specificity for binding substrates or E3s. Notably, these four hypervariable regions are located on one broad surface surrounding the active-site cysteine. It has been hypothesized that the two insertions in the Ubc7 core domain (Fig. 1) may be critical for its role in targeting specific substrates (e.g. Mato2, Sec61p, and YscY) (8, 46, 47) for ubiquitin-dependent degradation. Although this hypothesis of a hypervariable surface contributing to substrate and/or E3 specificity may prove correct, functional evidence is still lacking. In contrast, functional evidence presented here demonstrates that surfaces away from the ubiquitin-accepting cysteine, and thus away from the surface containing the hypervariable regions, are critical for determining the substrate specificity of the rat UBC4 isoforms. This involvement of a large surface of the E2 (Figs. 7 and 8) in determining substrate selectivity is an attractive concept as it can explain how small E2 molecules can encode such a diverse range of specificities.
Significantly, these results represent the first detailed mutagenesis of an E2 molecule related to substrate specificity. Unlike most studies of structure-function relationships that use mutagenesis to create artificial mutants with distinct properties, our studies have been based on naturally occurring isoforms. Thus, the different biochemical phenotypes based on these four critical residues are likely to be biologically important. The existence of such highly similar isoforms in the same tissue may not be redundant, but rather may permit fine regulation of conjugation of ubiquitin to specific substrates. The precise induction of UBC4-testis at the round spermatid stage of spermatogenesis would also argue for a specific function of this isoform (30). Inactivation of this gene in the mouse is currently underway and will likely yield further insights into the determinants of function present in the core domains of class I E2s.

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REFERENCES
1. Ciechanover, A. (1994) Cell 79, 13–21
2. Hochstrasser, M. (1996) Annu. Rev. Genet. 30, 405–439
3. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847
4. Hershko, A., Ganoth, D., Pehrson, J., Palazzo, R. E., and Cohen, L. H. (1991) J. Biol. Chem. 266, 16376–16379
5. King, R. W., Peters, J.-M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirsch-Vallabry, J. V. (1996) Science 272, 2138–2138
6. Pause, S., Bedard, N., Morales, C., Hingamp, P., and Trasler, J. (1996) Mol. Cell. Biol. 16, 4064–4072
7. Wing, S., and Jain, P. (1995) Biochemistry 34, 1257–1268
8. Chen, P., Johnson, P., Sommer, T., Jentsch, S., and Hochstrasser, M. (1993) Biochem. Biophys. Res. Commun. 195, 198–204
9. Treier, M., Staszewski, L. M., and Bohmann, D. (1994) Cell 78, 787–788
10. Scheffner, M., Werners, B. A., Hubrigtse, J. M., Levine, A. J., and Howley, P. M. (1990) Cell 63, 1129–1136
11. Ciechanover, A. (1994) Cell 79, 13–21
12. Haas, A. L., Warms, J. V. B., Hershko, A., and Rose, I. A. (1982) J. Biol. Chem. 257, 2543–2549
13. Haas, A. L., and Siepmann, T. J. (1997) FASEB J. 11, 1257–1268
14. Reiss, Y., Keller, H., and Hershko, A. (1989) J. Biol. Chem. 264, 10378–10383
15. Skowron, D., Craig, K. L., Tyers, M., Elledge, J., and Harper, J. W. (1997) Cell 91, 209–219
16. Feldman, R. M., Correll, C. C., Kaplan, K. B., and Deshaies, R. J. (1997) Cell 91, 221–230
17. Scheffner, M., Nuber, U., and Huijbregtse, J. M. (1995) Nature 373, 81–83
18. Huijbregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2563–2567
19. Jentsch, S., McGrath, J. P., and Varshavsky, A. (1987) Nature 329, 131–134
20. Seufert, W., and Jentsch, S. (1990) EMBO J. 9, 545–550
21. Jentsch, S., Seufert, W., Sommer, T., and Reins, H. A. (1990) Trends Biochem. Sci. 15, 195–198
22. Goebel, M. G., Yochem, J., Jentsch, S., McGrath, J. P., Varshavsky, A., and Byers, B. (1988) Science 241, 1331–1335
23. Nuber, U., Schwarz, S., Kaiser, P., Schneider, R., and Scheffner, M. (1996) J. Biol. Chem. 271, 2785–2800
24. Matuschewski, K., Hauer, H.-P., Treier, M., and Jentsch, S. (1996) J. Biol. Chem. 271, 2789–2794
25. Aristarkhov, A. E., Whitewing, H., and Radmer, J. V. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4248–4259
26. Sung, P., Prakash, S., and Prakash, L. (1988) Genes Dev. 2, 1476–1485
27. Morrison, A., Miller, E. J., and Prakash, L. (1988) Mol. Cell. Biol. 8, 1179–1185
28. Biederer, T., Volkwein, C., and Sommer, T. (1996) EMBO J. 15, 2069–2076
29. Byers, B. (1988) Science 241, 1331–1335
30. Wing, S., and Jain, P. (1995) Biochemistry 34, 1257–1268
31. Wing, S. S., Bedard, N., Morales, C., Hingamp, P., and Trasler, J. (1996) Mol. Cell. Biol. 16, 4064–4072
32. Cook, W. J., Jeffrey, L. C., Xu, Y., and Chau, V. (1993) Biochemistry 32, 13809–13817
33. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) Nucleic Acids Res. 16, 7351–7367
34. Scheffner, M., Hubrigtse, J. M., Vierstra, R. D., and Howley, P. M. (1993) Cell 75, 485–495
35. Haas, A. L., and Rose, I. A. (1982) J. Biol. Chem. 257, 10329–10337
36. Huibregtse, J. M., Scheffner, M., and Howley, P. M. (1993) Mol. Cell. Biol. 13, 775–784
37. Muller, D., Rehbein, M., Baumeister, H., and Richter, D. (1992) Nucleic Acids Res. 20, 1471–1475
38. Ciechanover, A., Heller, H., Elias, S., Haas, A. L., and Hershko, A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1365–1368
39. Hershko, A., and Heller, H. (1985) Biochem. Biophys. Res. Commun. 128, 1079–1086
40. Deleted in proof
41. Cook, W. J., Jeffrey, L. C., Sullivan, M. L., and Vierstra, R. D. (1992) J. Biol. Chem. 267, 15116–15121
42. Tong, H., Hateboer, G., Perrakis, A., Bernards, R., and Sixma, T. K. (1997) J. Biol. Chem. 272, 23813–23817
43. Gorod, P. A., and Vierstra, R. D. (1993) J. Biol. Chem. 268, 955–960
44. Hatakeyama, S., Jensen, J. P., and Weissman, A. M. (1997) J. Biol. Chem. 272, 15085–15092
45. Roest, H. P., van Klaveren, J., de Wit, J., van Gurp, C. G., Koken, M. H. M., Vermey, M., van Rojen, J. H., Hogerbrugge, J. W., Vreeburg, J. T. M., Baarends, W. M., Boutea, D., Groote, J. A., and Hoejmakers, J. J. H. (1996) Cell 86, 799–810
46. Biederer, T., Volkwein, C., and Sommer, T. (1996) EMBO J. 15, 2069–2076
47. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) Science 273, 1725–1728