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Substrate Specificity of Deubiquitinating Enzymes: Ubiquitin C-Terminal Hydrolases†

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ABSTRACT: Ubiquitin C-terminal hydrolases (UCH) are deubiquitinating enzymes which hydrolyze C-terminal esters and amides of ubiquitin. Here we report the processing of a number of ubiquitin derivatives by two human UCH isozymes (isozymes L1 and L3) and find that these enzymes show little discrimination based on the P1′ amino acid, except that proline is cleaved slowly. Ubiquitinyllysine derivatives linked by the α- or ϵ-amino group are hydrolyzed at identical rates. Isozyme-specific hydrolytic preferences are only evident when the leaving group is large. The ubiquitin gene products can be cotranslationally processed by one or both of these UCH isozymes, and purified UbCEP52 can be hydrolyzed by UCH isozyme L3. Binding of nucleic acid by UbCEP52 converts it to a form resistant to processing by these enzymes, apparently because of the formation of a larger, more tightly folded substrate. Consistent with this postulate is the observation that these enzymes do not hydrolyze large ubiquitin derivatives such as N^{ϵ}-ubiquitinyl-cytochrome-*c*, N^{ϵ}-^{K48}polyubiquitinyl-lysozyme, or an N^{α}-ubiquitinyl-β-galactosidase fusion protein. Thus, these enzymes rapidly and preferentially cleave small leaving groups such as amino acids and oligopeptides from the C-terminus of ubiquitin, but not larger leaving groups such as proteins. These data suggest that the physiological role of UCH is to hydrolyze small adducts of ubiquitin and to generate free monomeric ubiquitin from ubiquitin proproteins, but not to deubiquitinate ubiquitin−protein conjugates or disassemble polyubiquitin chains.

Ubiquitin is a 76-amino acid polypeptide found in all eukaryotes thus far examined. The covalent attachment of ubiquitin to a variety of cellular proteins is thought to target these proteins for intracellular degradation, and possibly other fates as well (*2*−*6*). A detailed description of the regulation of protein ubiquitinatination will require that we understand the enzymatic specificity of the enzymes which ubiquitinate proteins, as well as those that deubiquitinate them. The enzymes responsible for this posttranslational ubiquitination (E1, E2, and E3) have been reviewed extensively (*7*−*9*). The studies described here are aimed at describing the specificity of two deubiquitinating enzymes, the UCH-L1 and -L3 isozymes.

Although the broader consequences of protein modification by ubiquitin are just beginning to become apparent, the best understood role involves the ATP-dependent degradation of ubiquitinated protein. This degradative pathway is the major route for proteolytic removal of damaged, misfolded, and short-lived proteins and is necessary for the generation of MHC class I peptides for extracellular antigen presentation (see the reviews above). Many important regulatory proteins are also degraded by the ubiquitin system, probably including those involved in chromosome segregation, chromatin structure, DNA repair, cell cycle progression, tumor suppression, signal transduction, protein and peptide transport, antigen presentation, and transcriptional control.

Metabolism of Polymeric Ubiquitin. Ubiquitin-dependent protein degradation requires attachment of at least one ubiquitin to a target protein via an isopeptide bond between the C-terminal glycine of ubiquitin and an ϵ-amino group of lysine on the target protein. In many cases, additional ubiquitin molecules can be condensed to the first using K48 on the proximal ubiquitin (i.e., the one closest to the target protein) and the C-terminus of the more distal ubiquitin[1] (*10*). The polyubiquitinated protein may then be bound by the proteasome where the attached protein is degraded. Other forms of polyubiquitin (linked through K6, K11, K29, and K63) may also be present in the cell (*11*−*13*), but these are poorly defined in terms of their structure, formation and

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[1] Polymeric ubiquitin derivatives are named as follows. The polyprotein ubiquitin gene product (UBI4p in yeast) is referred to as proubiquitin. The products of the UBI1, -2 and -3 genes in yeast are referred to as ubiquitin C-terminal extension proteins (UbCEP). The length of the CEP can be added as a suffix; i.e., UbCEP52 or UbCEP76 in yeast. When the C-terminal carboxyl group of ubiquitin is involved in an amide bond, it is referred to as the ubiquitinyl group (Ub). The amino component of this amide bond can be contributed by either the amino terminus of a peptide (N^{α}-ubiquitinyl-peptide) or the ϵ-amino group of lysine (N^{ϵ}-ubiquitinyllysine). Where known, the number of the specific lysine in a peptide can be specified as a superscript prefix. Thus, a K48-linked ubiquitin dimer is referred to as N^{ϵ}-ubiquitinyl-^{K48}Ub. A larger polymer of ϵ-linked ubiquitin is referred to as polyubiquitin, with the identity of the specific lysine involved specified as a superscript prefix (i.e., ^{K48}polyubiquitin, ^{K63}polyubiquitin).

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Table 1: C-Terminal Extensions of the Proubiquitin Gene Product in Various Organisms[a]

extension	organism (no. Ub repeats)
-AF	*Acetabularia cliftonii (9)*
-C	*Bos taurus (4), Homo sapiens (3)*
-DI	*Caenorhabditis elegans (11)*
-DF	*Petroselinum crispum (6)*
-F	*Geodia cyndonium (6), Nicotiniana sylvestris (6), Pisum sativum (5), Arabidopsis thaliana (5),Glycine max (4), Antirhinium majus (>3), Sus scrofa (>3), Candida albicans (3), Euplotes eurystomus (3),*
-IQA	*Drosophila melanogaster (3)*
-K	*Hordeum vulgare (>2)*
-L	*Dictyostelium discoideum (5 & 3), Trypanosoma brucei brucei (1)*
-M	*Aglaothamnion neglectum (6)*
-N	*Dictyostelium discoideum (7 & 5), Gallus gallus (3), Phytophtora infestans (3)*
-Q	*Strongylocentrotus purpuratus (10), Zea mays (7), Oryza sativa (6), Tetrahymena pyriformis (5), Avena fatua (4), Neurospora crassa (4)*
-TQTSGKTFMTELTL	*Artemius nauplius (>2)*
-VYASPIF	*Cavia porcellus (4)*
-V	*Homo sapiens (9)*
-Y	*Cricetulus griseus (5), Gallus gallus (4), Mus musculus (4)*

[a] The proubiquitin[1] genes of most organisms encode head-to-tail repeats of the ubiquitin coding sequence with an additional amino acid or peptide at the C-terminus. A wide variety of residues must be cleaved from the polyubiquitin gene containing a variable number of ubiquitin repeats. Hence, the activity responsible for this cleavage are expected to show little P1′ specificity. Note the absence of proline at the junction. (This information was extracted from public sequence databases and is illustrative, not necessarily comprehensive.)

degradation rates, steady-state level, or function. The roles of these alternate ubiquitin isopeptide linkages are unknown. At least half of the cellular ubiquitin is found in these polymeric forms under most circumstances. The polyubiquitin chain must be disassembled by hydrolytic activities (*14*) during or directly after proteolysis, regenerating free monomeric ubiquitin.

The ubiquitin gene products also represent a source of polymeric ubiquitin. The stress-inducible proubiquitin gene (*ubi4* in yeast) encodes multiple copies of ubiquitin appended head-to-tail in a polyprotein (*15*, *16*) referred to a proubiquitin.[1] Each copy of ubiquitin must be released by accurate processing of the G76-M1 amide bond at the junction. The C-terminus of the proubiquitin is capped with an additional amino acid or oligopeptide, which varies among species (Table 1). The extension may prevent the activation of the C-terminus of this proprotein by the E2 conjugating enzymes (*17*) and thus prevent the inappropriate conjugation of this polymer to other cellular proteins. Two other types of ubiquitin genes also exist, those encoding ubiquitin carboxyl terminal extension proteins.[1] These fusion proteins consist of an N-terminal copy of ubiquitin fused to a ribosomal protein and are processed to give ubiquitin and the corresponding C-terminal extension protein (*18*, *19*). In rat, processing of UbCEP52 yields ubiquitin and the ribosomal protein L40 and processing of UbCEP80 yields ubiquitin and the ribosomal protein S27a (*20*). Ribosome biogenesis has been shown to require accurate hydrolytic processing of the UbCEP proproteins to their monomeric components, apparently before insertion into the ribosome (*19*).

Thus, all the ubiquitin proproteins are translated as fusion proteins and are processed to give the monomeric components. The need to process ubiquitin from multiple protein conjugates, natural gene fusions, and amino acid and peptide extensions of ubiquitin may explain the need for multiple deubiquitinating enzyme activities of the cell.

Deubiquitinating Enzymes. The generation of ubiquitin from proproteins and the processing of polyubiquitin chains to release monomeric ubiquitin are essential for the continued function of this important system. Collectively, the enzymes which catalyze such processing are called deubiquitinating enzymes (*5*, *21*). Two families of deubiquitinating enzymes have been identified: the ubiquitin-specific processing proteases (UBP)[3] and the ubiquitin C-terminal hydrolases (UCH).

Ubiquitin-specific processing proteases (UBP) were first identified and cloned using a screen for cleavage of large model ubiquitin fusion proteins (*22*, *23*). Three recombinant UBP enzymes from yeast (UBP1, -2, and -3) were assayed for cleavage of an N^{α}-ubiquitinyl-β-galactosidase fusion protein and other substrates. These enzymes hydrolyze ubiquitin fusion proteins and also can cotranslationally process the ubiquitin proproteins; however, quantitative rates for these processes are not available. Many additional homologous sequences have been identified from yeast to man, including those involved in tumorigenesis (*24*), in development and embryogenesis (*25*), in transcriptional silencing (*26*, *27*), and in growth control and cytokine response (*28*, *29*).

Another family of deubiquitinating enzymes are the ubiquitin C-terminal hydrolases (UCH), deubiquitinating enzymes which hydrolyze carboxyl-terminal esters and amides of ubiquitin (*30*). At least three mammalian isozymes of molecular mass near 25 kDa have been identified (*31*). The expression of these enzymes is tissue-specific (*32*) and developmentally regulated (*33*). The active site of these enzymes contains a catalytic triad consisting of cysteine, histidine, and aspartate (*34*) and utilizes a chemical mechanism similar to papain. UCH enzymes bind ubiquitin tightly, with a submicromolar K_m and K_d (*31*, *34*). Recently, the crystal structure of one of these enzymes has been described (*47*) although little is known about the natural

[2] The nomenclature used here is adapted from that of Schechter and Berger (*1*). From the N-terminus, amino acids of the substrate are abbreviated as ...P3−P2−P1−P1′−P2′−P3′.... The scissile bond is that between the P1 and the P1′ residue. The corresponding sites on the enzyme are labeled ...S3−S2−S1−S1′−S2′−S3′....

[3] Abbreviations: UBP, ubiquitin-specific processing protease; UCH, ubiquitin C-terminal hydrolase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

substrates and hydrolytic specificities of most UCH enzymes. Interestingly, members of this deubiquitinating family are also important modulators of cellular function. The *Aplysia* homologue to the human neural-specific UCH appears to be required for long-term potentiation of memory (*35*). Recently an 82 kDa UCH which enhances the growth suppression activity of BRCA1 has been identified (*36*). All these hydrolases are enzymatically active on a generic substrate, ubiquitin ethyl ester.

The specificities of deubiquitinating enzymes are not well understood. Papa and Hochstrasser (*24*) have suggested that the yeast UBP *Doa4* is involved in the hydrolysis of remnant peptide from polyubiquitin chains, acting late in the degradative pathway after proteolysis has occurred. The specificity of another member of the UBP family, isopeptidase T, has been extensively studied and has been shown to act on ϵ-linked polyubiquitin chains (*14*, *37*, *38*). Other substrates are poorly cleaved by isopeptidase T, including proubiquitin and the two UbCEP proproteins (*14*). These studies on substrate specificity have led us to the identify the yeast isopeptidase T homologue and verify its in vivo role as the primary enzyme responsible for disassembling polyubiquitin chains (*39*). To further define the specificity and the in vivo roles of UCH isozymes, we have tested natural and semisynthetic ubiquitin derivatives as substrates, with specific emphasis on their potential role in ubiquitin proprotein and polyubiquitin processing. The results suggest that human UCH isozymes L1 and L3 are apparently involved in processing of proubiquitin gene products and small molecular weight ubiquitin adducts, but not larger derivatives of ubiquitin.

EXPERIMENTAL PROCEDURES

Materials. Ubiquitin C-terminal hydrolases were prepared as described previously (*34*). All chemicals were reagent grade or better. Restriction endonucleases and DNA modification enzymes were from New England Biolabs. Recombinant human ubiquitin was expressed in *Escherichia coli* and purified as described below.

Subcloning of Proprotein Genes. The human UbCEP52 and UbCEP80 and the *S. cerevisiae ubi4* proubiquitin genes were excised from pSP72 cloning vector (*40*) by digestion with *Eco*RV and *Kpn*I. The cassette was ligated to a 5′ *Nde*I site (Klenow polymerase blunted) and the 3′ *Kpn*I site of the prokaryotic expression vector pRSET B (Invitrogen) with T4 DNA ligase. After transformation of the ligation mixture into Top 10 F′ competent *E. coli* (Invitrogen), clones were grown for DNA miniprep and assayed by restriction digestion with *Sca*I and *Xho*I (UbCEP52) or *Sca*I and *Bam*HI (UbCEP80). Correct recombinant plasmids were amplified and stored at −20 °C in TE buffer (*41*). The yeast proubiquitin gene was similarly inserted into the Klenow-blunted pRSET using *Eco*RV and *Hin*DIII, and colonies were screened with *Xho*I restriction digests of the isolated plasmids. These ubiquitin proprotein expression plasmids were named pRSUb52, pRSUb80, or pRSyUb5, respectively.

Purification of Ubiquitin Proproteins. The *E. coli* host strain BL21(DE3) (Invitrogen) was transformed with the appropriate expression vectors described above. For UbCEP proteins, the strain BL21(DE3)pLysE was used. Individual colonies were inoculated into 200 mL of LB media (*41*) supplemented with ampicillin (50 μg/mL) and grown overnight at 37 °C. This culture was used to inoculate 2 or 12 L of LB media. When the optical density (600 nm) of the cultures reached 0.45 (UbCEP) or 0.6 (yUb5), IPTG was added to 0.3 mM, and the cultures were grown for an additional 3 h. The cells were pelleted at 4000 rpm in an RC-3 rotor. Lysozyme was added to 0.1 mg/mL, and the bacteria were incubated for 30 min at 37 °C, sonicated, and recentrifuged as above. UbCEP52 was purified from the supernatant as described previously (*40*), with additional purification over a 300 mL Sephadex G-75SF gel filtration column and MonoS FPLC (Pharmacia).

Recombinant yeast proubiquitin was expressed in *E. coli* and purified by a modification of Jonnalagadda et al. (*42*). The bacteria were harvested by centrifugation, suspended in 50 mM Tris-Cl, pH 7.8, 1 mM EDTA, and sonicated (Heat Systems). After centrifugation for 30 min at 15000*g*, the supernatant was raised to 65 °C for 5 min and centrifuged again as above. The resulting heat stable supernatant was made 85% saturated in ammonium sulfate, stirred gently overnight at 4 °C, and centrifuged for 30 min at 10000*g* in a GSA rotor. The pellet was resolubilized in a minimal volume of water and, after lowering its pH to 4.6 with 1 M acetic acid, was applied to an FPLC Mono S 5/5 column (Pharmacia) in 50 mM NaOAc, pH 4.5. Ubiquitin oligomers were eluted in a linear gradient of 0−550 mM NaCl. Oligomers which cross reacted with anti-ubiquitin polyclonal antibodies (Accurate Scientific) eluted at 150, 200, 290, 350, and 400 mM NaCl ($n = 1-5$ ubiquitins, respectively). The pooled fractions were dialyzed against 10 mM Tris-Cl, pH 7.6, and concentrated by ultrafiltration. The preparation was homogeneous as judged by Coomassie-stained SDS−PAGE.

Purification of Truncated Ubiquitin Gene Products. To study P′ specificity, the truncated ubiquitin gene products Ub-CEP52$^{1-10}$, Ub-CEP80$^{1-10}$, and Ub-Ub$^{1-10}$ were prepared. Vectors encoding ubiquitin fused to the first 10 residues of CEP52 (Ub-IIEPSLRQLA), CEP80 (Ub-GKKRKKKVYT), or Ub (Ub-MQIFVKTLTG) were a generous gift of Dr. Martin Rechsteiner. Bacteria harboring the expression plasmids were grown to an A_{600} of 0.6, and induced for protein production with 0.5 mM IPTG. Supernatants were made as above but with 10 mM DTT in the buffer. The supernatants containing Ub-CEP52$^{1-10}$ or Ub-CEP80$^{1-10}$ were heat treated at 86 °C for 5 min, cooled to 4 °C, and centrifuged at 3500*g* for 15 min. In most cases, the supernatant was chromatographed on a 1 L column of G-100 superfine (Pharmacia). The supernatant containing Ub-Ub$^{1-10}$ was pretreated with 2.5% perchloric acid and centrifuged. The acid-soluble supernatant was subjected to gel filtration as above. In all cases, the fusion proteins obtained were homogeneous as judged by Coomassie-stained SDS−PAGE.

Preparation of Ub-Amino Acid Extension Proteins. A vector library encoding a variety of single amino acids C-terminal to ubiquitin was constructed using the polymerase chain reaction. To create this amino acid library at position 77, the coding region of the pRSUb80 vector (see above) was amplified with a degenerate 3′ primer which contained all possible codons followed by a stop codon and a *Hin*D III site. The primer sequences were 5′-atccatatgcagatcttcg-3′ and 5′-caagcttctaNNNaccaccacgaagtc-3′. The PCR products from this reaction were subcloned en masse into pCRII

(Invitrogen), and 40 minipreps were prepared. Inserts were present in 25 of the 40 minipreps and these inserts were sequenced (*43*). Clones were identified which encoded D, H, K, P, S, or T at the C-terminus. These were subcloned into pRSET using their *Nde*I and *Hin*DIII sites. Proteins were expressed and purified by heat denaturation and gel filtration, as described above. One additional clone was recovered due to a deletion in the PCR product. This frameshift resulted in a vector encoding N^{α}-ubiquitinyl-PRSLDSC, which was also expressed and purified.

Cotranslational Processing. The kanamycin resistance gene was incorporated into plasmids encoding UCH-L1 or UCH-L3 by insertion of a DNA cassette from pUC4K (Pharmacia). pRSUCH plasmids were digested with *Eco*RI and calf intestinal phosphatase. The *kan*^{r} gene cassette was excised from pUC4K with *Eco*RI. After gel purification of the insert and vector fragments, they were ligated, transformed into Top 10 F′ competent *E. coli*, and plated onto LB-kanamycin agar plates. The correct transformants were identified by the presence of a unique *Sca*I site in the *kan*^{r} cassette, and the *amp*^{r} gene was subsequently disabled by excision of an *Ava*II fragment in its center, followed by religation.

To coexpress enzymes and putative substrates in the same cell, BL21(DE3) cells harboring either the pRSyUb5, the pRSUb52, or the pRSUb80 plasmid (*amp*^{r}) were transformed with a pRSUCH plasmid (*kan*^{r}) and plated on LB agar containing both kanamycin and ampicillin to select for cotransformants. Induction with IPTG resulted in coexpression of the selected UCH isozyme along with a putative substrate. Processing was assessed by adding SDS−PAGE sample buffer directly to cell pellets and analyzed by Western blotting using antibodies specific for each substrate.

Other Substrates. A plasmid encoding a Ub-R-β-galactosidase fusion protein was a generous gift from Dr. Alex Varshavsky (pKKUbRβGal). Synthesis of Ub-R-β-gal was induced as described previously, and the fusion protein was purified as described for UCH (see above), except that the anion-exchange resin was eluted with 50 mM Tris-HCl, pH 7.5, and containing 150 mM NaCl. This resulted in significantly purified protein preparation (>80% homogeneous) which was used in gel and HPLC assays of fusion protein processing.

K48-linked diubiquitin (N^{ϵ}-ubiquitinyl-^{K48}Ub) was synthesized in vitro by incubation of human recombinant or bovine ubiquitin (Sigma) with the activating and conjugating enzymes of the ubiquitin system (*17*). Incubations contained 50 mM Tris-Cl, pH 8.0, 2 mM ATP, 5 mM $MgCl_2$, 5 mM phosphocreatine, 0.3 units/mL phosphocreatine kinase, 0.3 U/mL inorganic pyrophosphatase, 10 μg/mL ovalbumin, 30 μM E2−25k (plasmid a gift of Cecile Pickart), 0.1 μM E1 from rabbit liver (A. L. Haas, unpublished), and 5−10 mg/mL ubiquitin. Reaction mixtures were incubated at 37 °C for 40 min. The E1 and E2 enzymes were removed by passing the reaction mixture over a Mono Q anion exchange column (Pharmacia) at pH 7.6. Polyubiquitin chains[1] were purified by chromatography on Mono S FPLC (Pharmacia) as described above for proubiquitin.

An ϵ-linked ubiquitin dimer missing the C-terminal glycylglycine (N^{ϵ}-ubiquitinyl-^{K48}Ub$^{1-74}$) was synthesized as described above for N^{ϵ}-ubiquitinyl-^{K48}Ub, except that 6 mg/mL of des-Gly-Gly-ubiquitin was reacted with 2 mg/mL of native ubiquitin. The reaction was incubated at 37 °C overnight. Progress of the synthesis was assayed with HPLC and terminated by the method outlined above. Under these conditions, polyubiquitin chains are <97% terminated with des-Gly-Gly-Ub. The reaction products were separated on Mono S FPLC (Pharmacia).

N^{ϵ}-Ubiquitinyl-L-lysine and N^{ϵ}-ubiquitinyl-^{K48}Ub-L-lysine derivatives were synthesized as above except that the reactions included 200−500 mM of the particular lysine derivative, either N^{α}-acetyl-L-lysine (500 mM), N^{ϵ}-acetyl-L-lysine (500 mM), L-lysine (200 mM), or N^{α}-acetyl-L-lysine-*N*-methyl amide (200 mM). These reactions were allowed to incubate overnight at 37 °C to ensure maximal lysine conjugation. C-8 Reverse phase HPLC was used to monitor these reactions, and the reactions were terminated as described above.

Hydrolysis Studies. Hydrolysis rates were measured by incubating the above substrates with homogeneous UCH-L1 or -L3. Conditions for assay were essentially as described previously (*44*). Incubation of UCH was performed at 37 °C in 50 mM Tris-Cl, pH 7.6, with 5 mM DTT and 50 μg/mL ovalbumin, for varying amounts of time. Substrate concentrations were 15 μM, approximately 20-fold higher than the K_m for ubiquitin ethyl ester. Values are reported as the mean and the standard error of the mean for between 6 and 30 determinations. In cases where no catalysis was observed, the substrate was raised to its highest possible concentration.

RESULTS

P1′ Specificity. Removal of a single amino acid or small peptide from the C-terminus of ubiquitin must occur during processing of ubiquitin precursors and metabolites (Table 1). As ubiquitin ethyl ester (*44*) and Ub-DTT (*45*) are both rapidly hydrolyzed by UCH isozymes, it was of interest to determine if these enzymes exerted any specificity for residues at the P1′ position of ubiquitin fusion proteins.[2] Such specificity might manifest itself in differential rates of cleavage of α-linked amino acid extensions. Figure 1 shows the hydrolysis rates obtained with UCH-L1 and -L3 isozymes for Ub-Pro, Ub-Lys, Ub-His, and Ub-Asp, relative to ubiquitin ethyl ester, our generic reference substrate. The data show that neither UCH isozyme exhibited a strong preference for the P1′ residue (*1*) immediately following ubiquitin, except when it was proline. Ub-amino acid extensions were hydrolyzed by both UCH isozymes at rates only 1−2 orders of magnitude more slowly than UbOEt, whereas Ub-Pro was hydrolyzed at about 3 or 5 orders of magnitude more slowly than UbOEt (Table 2). These rates were determined at 15 μM substrates and probably represent V_{max} values (but see Discussion). Thus, these UCH isozymes are not selective with respect to the charge or size of residues at the P1′ position when the ubiquitin extension is a single non-proline amino acid residue.

Comparison of Peptidase and Isopeptidase Activities. Because ubiquitin is also conjugated to proteins through an isopeptide bond (i.e., through the ϵ-amino group of lysine), it was of interest to examine whether UCH isozymes could cleave Ub-ϵ-amino lysine derivatives. It has been shown that UCH-L3 can hydrolyze both types of bonds (*46*), although absolute rates were not determined. As a model

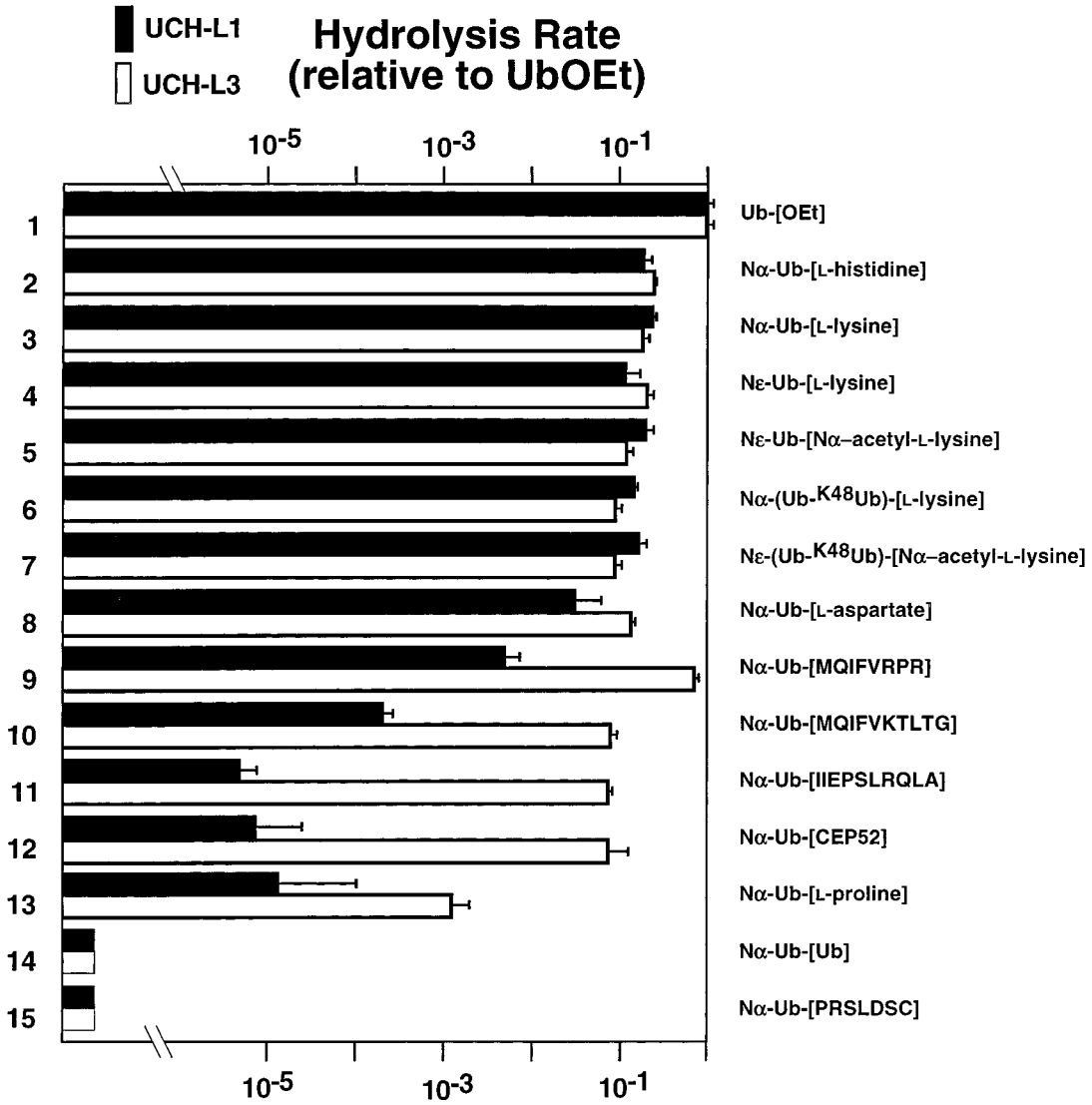

FIGURE 1: Relative rates of hydrolysis of ubiquitin derivatives by UCH isozymes. The rates of hydrolysis were measured by HPLC according to the method Wilkinson et al. Brackets ([ ]) surround the leaving group. The rates shown are obtained with 15 μM substrates (~20 times K_m) and are given as the ratio of rates for the indicated substrate vs that for ubiquitin ethyl ester. The error bars represent the standard error of the mean. (See Table 1 for absolute rates.) Note the log scale.

isopeptidase substrate, we synthesized N^{ϵ}-ubiquitinyl lysine by incubation of ubiquitin and lysine with the E1 activating enzyme, the E2-25K conjugating enzyme, and ATP. In this synthesis, the excess lysine nucleophile captures the thiol-esterified ubiquitin from the transient E2-Ub intermediate, forming exclusively the N^{ϵ}-ubiquitinyllysine product and halting further synthesis of polyubiquitin by E2-25k. Both UCH isozymes rapidly hydrolyzed N^{ϵ}-linked lysine (Figure 1). Additionally, the rates were essentially identical to those obtained with N^{α}-linked lysine (Table 2).

A more relevant ϵ-linked substrate might be an N^{ϵ}-ubiquitinated peptide similar to the degradation remnants expected to be generated by the action of the proteasome on ubiquitinated proteins. To more closely mimic a peptide bond at the α amino group of an N^{ϵ}-linked lysine, we synthesized and tested N^{ϵ}-ubiquitinyl(*N*-α-acetyl)lysine as a substrate. We found the addition of an acetyl functionality to the α-amino group did not affect the hydrolysis rate of N^{ϵ}-ubiquitinyllysine (Figure 1). Both isozymes cleaved acetylated and unacetylated substrates at a rate roughly 8−10-fold slower than the rate of cleavage of ubiquitin ethyl ester. Subsequent experiments showed that there was also no effect of amidating the carboxyl group of lysine with *N*-methylamine (data not shown).

Polyubiquitin Processing. Because N^{ϵ}-ubiquitinyllysine was a good UCH substrate, we sought to determine if N^{ϵ}-diubiquitinyllysine derivatives were good UCH substrates. If these enzymes function in the removal of a K48-linked remnant peptide from polyubiquitin, they should process lysine derivatives at the C-terminus of polyubiquitin chains. As a model substrate, we synthesized N^{ϵ}-(Ub-^{K48}Ub)lysine and N^{ϵ}-(Ub-^{K48}Ub)(*N*-α-acetyl)lysine. The lysine is removed from these polyubiquitin derivatives at rates identical to the simpler N^{ϵ}-Ub-lysine derivatives, regardless of the presence of a second ubiquitin (Figure 1). Neither UCH is able to hydrolyze the K48 isopeptide bond. Neither Ub-^{K48}Ub nor Ub-^{K48}Ub(des-Gly-Gly) is cleaved, even at a 4-fold molar excess of enzyme for 2 h at 37 °C (not shown). This hydrolysis rate is therefore more than 8 orders of magnitude slower than the ubiquitin esterase rate of either enzyme. This suggests that the ubiquitin binding site on UCH isozymes recognizes a face of ubiquitin distant from the K48 linkage

Table 2: Rates of Hydrolysis of Ubiquitin Derivatives by UCH-L1 and UCH-L3[a]

	activity (μmols/min/mg)			
	UCH-L1[b]		UCH-L3[b]	
substrate	mean	SEM	mean	SEM
1, Ub-[OEt]	30	6.0	110	22
2, N^{α}-Ub-[L-histidine]	6.0	0.9	26	2.6
3, N^{α}-Ub-[L-lysine]	7.2	0.7	20	4.0
4, N^{ϵ}-Ub-[L-lysine]	3.7	1.4	23	4.0
5, N^{ϵ}-Ub-[N^{α}-acetyl-L-lysine]	6.3	1.3	13	2.6
6, N^{α}-(Ub-^{K48}Ub)-[L−lysine]	4.7	0.3	9.9	2.2
7, N^{ϵ}-(Ub-^{K48}Ub)−[N^{α}-acetyl-L−lysine]	5.0	1.0	10	2.0
8, N^{α}-Ub-[L-aspartate]	9.9×10^{-1}	9.0×10^{-2}	15	2.4
9, N^{α}-Ub-[MQIFVRPR]	1.5×10^{-1}	6.3×10^{-2}	79	8.8
10, N^{α}-Ub-[MQIFVKTLTG]	6.0×10^{-3}	1.9×10^{-3}	8.8	1.7
11, N^{α}-Ub-[IIEPSLRQLA]	1.4×10^{-4}	6.6×10^{-5}	8.5	0.9
12, N^{α}-Ub-[CEP52]	2.1×10^{-4}	4.8×10^{-3}	8.4	5.9
13, N^{α}-Ub-[L-proline]	3.9×10^{-4}	2.5×10^{-3}	1.3×10^{-1}	8.8×10^{-2}
14, N^{α}-Ub-[UB]	$<1 \times 10^{-5}$		$<1 \times 10^{-5}$	
15, N^{α}-Ub-[PRSLDSC]	$<1 \times 10^{-5}$		$<1 \times 10^{-5}$	

[a] Rates of hydrolysis of the indicated substrates are shown. The leaving group is bracketed. The detection limit in this assay is about 1×10^{-5} μmol/min/mg. [b] The mean and the standard error of the mean were derived from between 6 and 30 replicate measurements.

site and suggests that UCH could function in generating a free C-terminus on polyubiquitin chains by the removal of small peptides and/or cellular nucleophiles (see Discussion).

Fusion Peptide Processing. It has been postulated that ubiquitin C-terminal hydrolases could participate in the processing of ubiquitin gene products. It is unlikely that a protein as small as UCH could exhibit specificity for ubiquitin and also a significant portion of the C-terminal extension. Thus, if UCH activity were responsible for processing ubiquitin gene products, then these enzymes would be expected to exhibit specificity for the peptide sequences at the junction between ubiquitin and the C-terminal extension. Model substrates synthesized to test this hypothesis consisted of ubiquitin followed by the first 10 amino acids of the C-terminal extensions, i.e., Ub-CEP52$^{1-10}$ (substrate 11, Figure 1) and Ub-Ub$^{1-10}$ (substrate 10, Figure 1).

Figure 1 shows that isozyme L3 exhibited little selectivity for any of the peptide extensions, cleaving them nearly as rapidly as it cleaves single amino acid extensions. This is also consistent with data which suggests that UCH-L3 has no difficulty cleaving a wide variety of peptide substrates from ubiquitin if the peptides are less than about 20 residues (Rechsteiner, M., and Wilkinson, K. D., unpublished). Interestingly, UCH-L1 exhibited considerably more specificity, showing rates of hydrolysis of these substrates that are over 2 orders of magnitude slower than the rates of L3-catalyzed hydrolysis (Table 2). Still, UCH-L1 exhibits notable selectivity; the Ub-Ub$^{1-10}$ substrate is hydrolyzed over 40-fold faster than the Ub-CEP$^{1-10}$ substrates by this enzyme.

Aside from the natural peptide sequences at the C-terminus of ubiquitin, we also created one other substrate. Ub-PRSLDSC, a ubiquitin-peptide fusion with proline at the P′ cleavage junction, was created by a PCR error that resulted in the read-through of the reading frame into the vector multicloning site. Neither enzyme was able to cleave this fusion peptide at a measurable rate, although UCH-L3 is able to cleave Ub-Pro. The hydrolysis rate of these peptide fusions was more than 7 orders of magnitude slower than that for UbOEt.

Ubiquitin Proprotein Processing. Because model substrates containing the first 10 residues of ubiquitin proproteins were hydrolyzed by UCH isozymes, we determined the rate of cleavage of full-length ubiquitin gene products by these enzymes. Purified α-linked Ub oligomers were very slow substrates for UCH-L1 and were not cleaved at all by UCH-L3 (Figure 1). Micromolar UCH-L1 was able to cleave N^{α}-diubiquitin at 37 °C in vitro with a half-life of 30 min (not shown). This corresponds to a rate of at least 6 orders of magnitude slower than for UbOEt. UCH-L1 is reported to exist at 1−2% of total soluble brain protein (*32*), so these assay conditions may be physiologically relevant (see Discussion).

The zinc-finger fusion proteins UbCEP52 and UbCEP80 are the two other natural ubiquitin proprotein substrates studied. High amounts (100 mU) of either recombinant UCH added to bacterial expression lysates for 2 h failed to hydrolyze UbCEP52 or UbCEP80 to their monomeric components, on the basis of immunoblotting of the expression lysates (not shown). Because UbCEP52 was more highly expressed than UbCEP80 and because the antibodies to CEP52 had a higher titer and were more specific than the anti-CEP80 antibodies, we chose to further characterize the UCH-catalyzed hydrolysis of the UbCEP52 protein.

Surprisingly, purified UbCEP52 was hydrolyzed by both enzymes, though the L3 isozyme catalyzed the reaction much more rapidly (Figures 1 and 2). The rate of processing of UbCEP52 by UCH-L3 approaches the rate of hydrolysis of the Ub-amino acid extensions, about 200 min^{-1}. To confirm the specificity of this reaction (reverse phase HPLC does not detect the basic extension protein), SDS−PAGE and immunoblotting were used to identify the products (Figure 2). The appearance of ubiquitin and CEP52 detected by SDS−PAGE is consistent with the rates measured by HPLC. UCH-L1 also hydrolyzed the substrate to a measurable degree, but the rate was 2.1×10^{-4} μmol/min/mg, or $\sim 10^{-5}$ the rate of ester hydrolysis.

The above results suggest that the bacterial lysates contain something which interferes with UbCEP52 hydrolysis, but not with UbOEt hydrolysis. UbCEP52 possesses a C_2H_2 zinc-finger binding motif, so we first examined whether

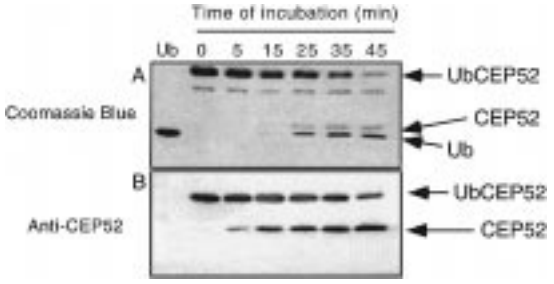

FIGURE 2: UbCEP52 is a substrate for UCH-L3. Each lane contains 10 μg of substrate and 1 μg of enzyme. The time of digestion is given in minutes. (A) SDS−PAGE of the reaction time course; protein detected by Coomassie Blue staining. (B) Immunoblot of a duplicate gel, probed with rabbit antisera to human CEP52. The unmarked band is a minor contaminant.

binding of zinc could inhibit the UCH-L3 hydrolysis of UbCEP52. $Zn(OAc)_2$ (10 mM) did not inhibit UbCEP52 hydrolysis by either enzyme (not shown). Whether the zinc-finger motif binds metal in vivo remains to be elucidated; however, addition of excess metal ion does not inhibit the processing of the proprotein by UCH.

The presence of a zinc-finger motif in a ribosomal protein is presumptive evidence of nucleic acid binding. To test if binding of nucleic acid inhibited processing, assays were performed in the presence of nucleic acids. In vitro addition of 50 μg/mL of either plasmid DNA or a double-stranded 26-base pair DNA cassette inhibited the hydrolysis of UbCEP52 by 50%, whereas a single-stranded 42-base pair oligodeoxynucleotide at the same concentration was only minimally effective (Figure 3). Whole yeast RNA was even better at inhibiting processing, showing 60−80% inhibition. Phenol/chloroform extraction of this RNA did not improve the processing, suggesting that the inhibition was not due to other contaminating proteins in the RNA preparation. Also, preincubation of the RNA with RNase A restored the UbCEP hydrolysis rate back to control rates. These results imply that the nascent proprotein can only be cleaved by UCH before nucleic acids are bound to the fusion peptide and that assembly into the ribosomal subunit would probably prevent processing.

UCH Isozymes Can Cotranslationally Process Ubiquitin Proproteins. Ubiquitin proproteins are very rapidly processed in vivo (*19*, *22*). The UCH isozymes appear to be very efficient at processing peptides from the C-terminus of ubiquitin, but not if the C-terminal extension has a chance to fold into a tight, globular domain (see above). Further, only UCH-L1 is able to rapidly process the proubiquitin precursor, and this isozyme is present at low levels in most tissues. These observations suggest that processing of some ubiquitin gene products may occur before folding or subunit assembly is completed. To test the idea that UCH isozymes can process UbCEPs cotranslationally, we cotransformed cells with vectors expressing UCH and Ub proproteins in various combinations. UCH-L1 was found to hydrolyze polyubiquitin (60%) and UbCEP80 (50%), but not the UbCEP52 (>5%) (Figure 4). These data are consistent with the above data from peptide hydrolysis, in that UCH-L1 prefers to hydrolyze ubiquitin-like peptides and also hydrolyzes the complete proubiquitin, albeit slowly. In contrast, UCH-L3 was found to hydrolyze both Ub-CEP fusions, but not proubiquitin (Figure 4).

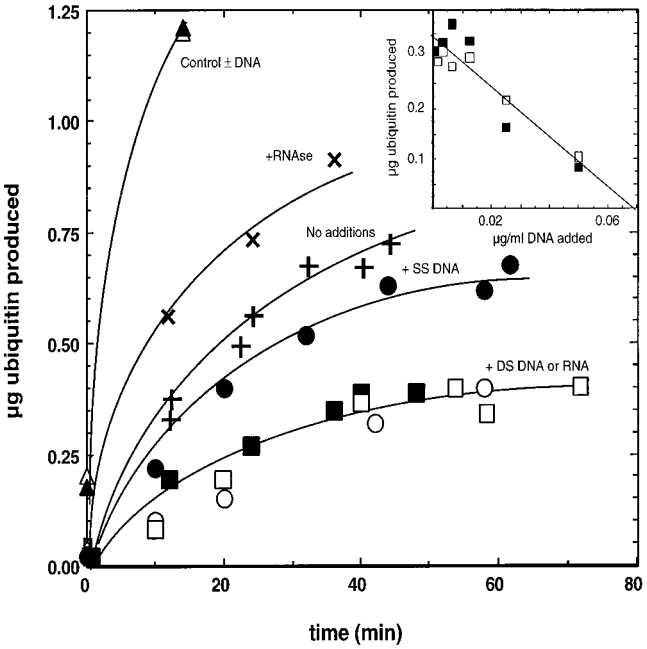

FIGURE 3: Nucleic acid inhibits the processing of UbCEP52 by UCH-L3. Nucleic acid was added at a concentration of 0.05 mg/mL and incubated for 10 min with the substrate before enzyme was added to start the reaction. Addition of dsDNA to UbOEt had no effect on the rate of ester hydrolysis (triangles). The rate of hydrolysis of UbCEP52 is only a few-fold slower (+). Addition of RNase A slightly increased the rate of hydrolysis of UbCEP52 (×). Single-stranded DNA had little effect (solid circles), while either *E. coli* RNA (solid squares), a plasmid DNA (open circles), or a double-stranded 42 bp DNA (open squares) were significantly inhibitory.

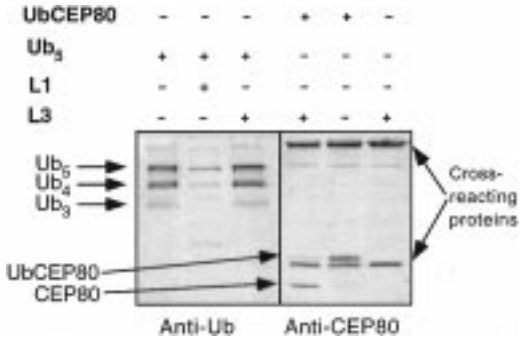

FIGURE 4: Cotranslational processing of the proubiquitin (left panel) and UbCEP80 (right panel) gene products by UCH-L1 and UCH-L3. The bacterial host BL21(DE3) was cotransformed with a plasmid encoding the substrates and the Amp^r gene product and a second vector encoding the indicated enzyme and Kan^r gene product. Protein production was induced with IPTG for 3 h and whole cell lysates were subjected to SDS−PAGE and immunoblotting with anti-ubiquitin (left panel) or anti-CEP80 (right panel) antibodies.

DISCUSSION

In an effort to understand the substrate specificity and the potential in vivo roles of deubiquitinating enzymes, we have examined the rates of hydrolysis of a series of natural and model substrates. Such studies can be important in suggesting possible phenotypes of mutant enzymes and the design of direct tests of the proposed roles using gene disruption and transgenic approaches. For instance, in vitro studies on the specificity of isopeptidase T, a UBP-type deubiquitinating enzyme, prompted us to propose that it was the major isozyme responsible for disassembling polyubiquitin chains

(*14*). This predicted that disruption of the yeast homologue UBP14 would lead to accumulation of free polyubiquitin chains. Recently we have confirmed this prediction and shown that mild proteolytic defects in these mutants stems from the accumulation of free chains (*39*). Further, specificity studies form a basis for the interpretation of the recently solved X-ray crystal structure of UCH-L3 (*47*). For the purposes of the discussion below, the protease nomenclature of Schechter and Berger (*1*) is adopted; sites to the N-terminal side of the scissile bond are named S1, S2, etc. as one progresses toward the N-terminus of the peptide, and S1′, S2′, etc. as one progresses to the C-terminus of the peptide. The corresponding amino acid residues on the substrate are indicated by P2, P1, P1′, P2′, etc. The substrate specificity and potential physiological roles are, in part, determined by the specificity of these binding sites.

This work describes the substrate specificity of two closely related UCH isozymes, UCH-L1 and -L3. The hydrolysis rates reported here were determined at 15 μM substrates, approximately the same concentration as that of total ubiquitin in the cell. The K_m for hydrolysis of ubiquitin ethyl ester is approximately 1 μM and is identical to the ubiquitin binding constant (*34*). Thus, in the absence of unfavorable interactions between the enzyme and the leaving groups, the measured rates would reflect V_{max} values. With some of the poorer substrates, however, the slower observed rates of hydrolysis may be due to higher K_m values for these substrates. With the exception of the peptide with proline at the junction, we have no evidence for this. If substrate binding were poor, the accumulation of ubiquitin should very effectively inhibit the hydrolysis of substrate and the time course of reaction should be markedly nonlinear. This was not observed. Also, at rates that are 3−4 orders of magnitude slower than the rate of Ub(OEt) hydrolysis the concentration of enzyme approaches the concentration of substrate. Thus, the interpretation of rates in terms of the steady-state assumption is not valid. Irrespective of the reasons for the slower rates of hydrolysis, it is clear that these differences are manifest at concentrations that are many times that observed in a cell and that the rates reported will overestimate the relative rates of hydrolysis that would pertain in vivo.

Ubiquitin Binding to the S Site(s). The available evidence suggests that the S sites form an extensive binding site for intact ubiquitin. The only demonstrated activity of UCH isozymes is for cleavage of amide and ester bonds at the C-terminus of ubiquitin. There is little or no affinity for small peptides at the C-terminus of ubiquitin (such as glycylglycine), but ubiquitin is bound with a micromolar binding constant (*34*). Ubiquitin aldehyde is a tightly bound inhibitor of these enzymes. Further, NMR measurements have confirmed an extensive area of contact between ubiquitin and UCH-L3, encompassing over 20% of the surface residues on ubiquitin (Wand, J., and Wilkinson, K. D., unpublished), including the C-terminus. This contact surface cannot include the N-terminus of ubiquitin, as a hexahistidine tag at the N-terminus has little or no effect on the rates of hydrolysis (data not shown). In agreement with this result, it has been shown that these enzymes bind to immobilized $(His)_6$-ubiquitin (*48*). The surface of ubiquitin containing K48 is also not in the S1 recognition site on ubiquitin, as N^{ϵ}-Ub-^{K48}Ub derivatives are good substrates for cleavage of the leaving group from the free C-terminus (Figure 1 and Table 2). N^{ϵ}-Ub-^{K48}Ub is not a substrate (see results), probably because the leaving group ubiquitin is tightly folded against the C-terminal face of the distal ubiquitin. Finally, the interactions between ubiquitin and UCH-L3 are predominantly ionic, as evidenced by the previously observed inhibition of binding and activity by salt (*34*).

S1′ Specificity. Many different amino acids and peptides are found as natural extensions of ubiquitin genes in eukaryotes (Table 1). Putative processing enzymes would have to either have broad specificity at the P1′ site or exhibit significant sequence variability from species to species in order to accommodate their respective species-specific leaving groups. In fact, UCH sequences are very similar across species, with rat, human, and bovine UCH-L1 being over 98% identical. Our results show that UCH isozymes exhibit very little specificity for the P1′ residue of ubiquitin substrates (Figure 1) with essentially identical rates with acidic, basic, or neutral leaving groups. If UCH isozymes were responsible for processing the amino acid extensions of ubiquitin gene products, they would exert little selective pressure on the nature of that leaving group. This may be why there seems to be little selective pressure to maintain the identity of this extension amino acid (see Table 1).

As both α- and ϵ-linked derivatives have to be processed from the C-terminus of ubiquitin, we examined the selectivity for cleavage of these two types of amide bonds. These enzymes exhibited little or no discrimination based on the identity of the amide bond to lysine (α vs ϵ), the charge at the other amine (free amine vs *N*-acetyl), or the charge at the carboxyl group (carboxyl vs *N*-methyl amide). Further, the same derivatives can be efficiently processed from the C-terminus of the polyubiquitin chain. Thus, at least with small leaving groups, these enzymes could be involved in processing both the amino acid and small peptide extensions of various gene products, as well as the N^{ϵ}-(poly)ubiquitinyllysine expected to be generated by the action of the proteasome on polyubiquitinated protein substrates.

The S′ Site(s) Will Not Bind Larger Protein Domains. Figure 1 demonstrates that UCH-L3 is generally able to hydrolyze a variety of small peptide fusions at the C-terminus of ubiquitin. To examine if there was any selectivity based upon P′ sequences, we have also measured the rates of processing of the ubiquitin gene products and short model substrates consisting of ubiquitin fused to the first 10 amino acids of the C-terminal domains.

UCH isozymes exhibit significant selectivity in the processing of the ubiquitin gene products. UCH-L3 is able to efficiently process the Ub-CEP52 gene product, but not the Ub-CEP80 or proubiquitin gene products. Isozyme L1 is only able to slowly process the proubiquitin gene product in vitro and in vivo. It has been reported that the yeast homologue YUH1 also exhibits a similar selectivity in that small fusion proteins can be efficiently processed, but not larger fusions (*49*). The drosophila homologue has been reported to be able to process N^{α}-ubiquitinyl-IκBα (314 amino acids), but not larger fusions (*50*).

Interestingly, we observed that nucleic acid binding to Ub-CEP52 prevented its processing by UCH-L3 (Figure 3). The addition of nucleic acid to UbOEt had no effect on its hydrolysis, suggesting that the nucleic acid was directly binding to Ub-CEP52 and causing a conformational change

which prevented processing. The binding of nucleic acid by Ub-CEP52 is not unexpected; the CEP domain contains a zinc-finger motif, the protein is a ribosomal subunit, and mutants in this gene are defective in rRNA processing (*19*). Nonetheless, we have not shown binding directly and these experiments are in progress.

The above results suggest that the selectivity of the S′ sites may be based on factors other than size. One factor could be the accessibility of the peptide bond at the C-terminus of ubiquitin. It might be expected that ubiquitin fusion proteins with significant mobility and flexibility at the junction could be good substrates while those that are more constrained (proline) and/or sterically restricted (large) would be poor substrates. This is consistent with the ligand-induced inhibition described above (i.e., binding of nucleic acid may cause a less mobile conformation around the Ub-CEP52 junction) as well as the restricted nature of the substrate binding cleft observed in the UCH-L3 crystal structure (*47*).

Substrate Specificity Based on the P′ Peptide Sequence. An alternative explanation for the observed selectivity in processing of ubiquitin gene products is that the enzymes may exhibit significant selectivity based on the amino acid sequences binding to the S′ sites. To examine the contribution of the P′ residues to the observed selectivity, we have used model substrates consisting of ubiquitin fused to small peptides, including the first 10 amino acids of each ubiquitin gene product. Figure 1 demonstrates that UCH-L3 is not very selective for the P′ residues, processing every small peptide tested except those containing proline at the scissile bond. This specificity is similar to that reported for the yeast UCH; i.e., ubiquitin extended by E, C, D, G, T, or M (but not P) was hydrolyzed efficiently (*49*). This may be because the secondary amine of the proline has a somewhat higher pK_a than the primary amino group in the peptide bond of most amino acids, or it may reflect a steric constraint imposed at the scissile bond. UCH-L3 is unable to process at proline in the Ub-PRSLDSC peptide fusion. It is likely that the presence of proline at the P1′ position "kinks" the peptide such that it cannot be accommodated in the active site cleft. Consistent with this we find that this fusion peptide does not inhibit hydrolysis of other substrates and probably does not bind (data not shown). The presence of a proline at position P4′ (Ub-CEP52$^{1-10}$, substrate 11) or P7′ (Ub-Ub$^{1-5}$-RPR, substrate 9) has little effect on the rate of peptide processing by UCH-L3, suggesting that the cleft may be considerably less restricted at that distance from the active site nucleophile. The Ub-Ub$^{1-10}$ construct is processed very effectively by UCH-L3, but the Ub-Ub fusion protein is not cleaved at all, reinforcing the conclusion that a tightly folded domain at the C-terminus of ubiquitin is not generally a substrate for these enzymes.

In contrast to the permissiveness of UCH-L3 processing, the processing by UCH-L1 is more selective, with ubiquitin-related peptide fusions being reasonable substrates and Ub-CEP52$^{1-10}$ being a poor substrate. While it is not clear whether this selectivity is due to subsite specificity at P1′−P3′, or the presence of proline in sites P4′−P7′, it is clear that this is a much more selective enzyme. This specificity may be related to interactions with an occluding loop which is postulated to form part of the S′ sites on the UCH family of enzymes (see below).

Cotranslational Processing. The above results demonstrate that there is considerable selectivity in the processing of ubiquitin gene products by these UCH isozymes. UCH-L3 appears to prefer processing of Ub-CEP gene products, while UCH-L1, is very selective for the proubiquitin gene product. There is, however, some question as to the physiological significance of these processing events, especially those catalyzed by UCH-L1, which occur at an extremely slow rate. This led us to ask if these enzymes might be involved in cotranslational processing. Normal processing is known to be extremely efficient, with no evidence for accumulation of intermediates in the process. Further, if these enzymes are involved in processing, they must act before significant assembly into ribosomal subunits, and/or folding of stable domains C-terminal to ubiquitin. When enzyme and substrate were coexpressed in *E. coli*, the efficiency of processing was high and the selectivity was similar to that observed above. UCH-L1 was able to process over 80% of the proubiquitin gene product, and little of the Ub-CEP gene products, while UCH-L3 was most efficient in processing the Ub-CEP fusion proteins (>50% processed). Thus, it appears that processing is much more efficient if the enzyme is present during the synthesis of the substrate. We have attempted to confirm this by demonstrating the association of UCH-L3 with polyribosomes synthesizing the substrates. When an in vitro transcription/translation system is supplemented with DNA encoding the substrate, endogenous UCH activity is found exclusively in the soluble fractions. Even upon addition of exogenous UCH isozymes, little or no UCH activity can be found stably associated with the ribosomes (data not shown). It may be that the association is only fleeting and unstable, or it may be that processing occurs after release of the substrate polyprotein from the ribosome but before folding of the complete protein.

Molecular Basis of Specificity. We have recently reported the X-ray crystal structure of UCH-L3 (*47*). The protein has a core catalytic structure that strongly resembles cathepsin B, a papain-like protease. The active site groove is occluded by two loops, and it is postulated that a substrate-induced conformational change is required to clear the cleft and allow access to the active-site cysteine. Thus, only ubiquitin derivatives are substrates because only they can form the extensive interactions with the S′ site required to trigger the necessary conformational change generating the active conformation of the enzyme.

Specificity for P′ residues must be determined by the residues lining the corresponding S′ sites on the UCH enzymes. The sequence of these proteins varies widely in several areas, including a region just N-terminal to the active site histidine. This sequence is disordered in the UCH-L3 structure, but may be positioned to form a significant contact region with the P′ residues of substrates (*47*). Thus, it is likely that this hypervariable region is important in determining substrate selectivity and the somewhat shorter loop near the active site cysteine in UCH-L1 restricts the possible substrates by conferring a narrower or more restricted active site cleft. These predictions could be tested by obtaining the structure of UCH-L1 and/or using site-directed mutagenesis and domain-swapping approaches.

Potential Physiological Roles for UCH Isozymes. The possible physiological roles for UCH isozymes are limited by the temporal and spatial patterns of expression of the

enzymes and putative substrates, as well as by restrictions imposed by the substrate specificity examined here. With respect to the former, there is a marked tissue specificity to the expression of UCH isozymes, with UCH-L1 being expressed at very high levels in neural and diffuse neuroendocrine tissues and UCH-L3 being expressed primarily in hematopoetic tissues (*32*). There is little evidence of temporal regulation, as these enzymes seem to be present in all stages of the cell cycle and both early and late in development. A third isozyme, UCH-L2 has been reported to be widely distributed, albeit at lower levels than either of the two isozymes studied here (*32*).

The distribution of putative substrates is more difficult to assess, although the results discussed above suggest that substrates will include the ubiquitin proproteins and small molecule adducts of ubiquitin. The latter are expected to be widely distributed, as there is extensive activation and conjugation of ubiquitin in all tissues examined. All of the intermediates in the enzymatic activation of the C-terminus of ubiquitin are thiol esters and they are effectively trapped by reaction with small molecular weight thiols and amines. There is a much more specific expression of ubiquitin proproteins. Rapidly growing cells express high levels of ubiquitin-ribosomal fusion proteins, while more differentiated cells (such as neurons) express ubiquitin primarily from the proubiquitin locus (*51*, *52*).

These considerations suggest that UCH-L1, the neuronal specific isozyme, may be more efficient at cleaving the proubiquitin precursor, while the hematopoetic specific UCH-L3 might prefer ubiquitin ribosomal fusion proteins as substrates. These predictions are borne out using ubiquitin fusion peptides as substrates. UCH-L1 is found at high levels only in neurons and diffuse neuroendocrine tissues, and it cleaves the proubiquitin model substrate (Ub-Ub$^{1-10}$, substrate **10**) much faster than it cleaves the ubiquitin ribosomal fusion protein model substrate Ub-CEP52$^{1-10}$ (substrate **11**). UCH-L3 on the other hand can cleave all the model substrates at a significant rate. The specificity of cotranslational cleavage of the full length gene products reflects the results with small peptide fusions, implying that a portion of the UCH specificity derives from interactions with P′ residues. Large, tightly folded leaving groups are not substrates for this class of enzyme, although there are differences in the selectivity demonstrated by each enzyme.

These results support the idea that UCH enzymes are responsible for cotranslational processing of the polymeric ubiquitin gene products and/or salvage of ubiquitin from small molecular weight adducts. Only ubiquitin derivatives will be substrates, probably because of the obligatory substrate-induced conformational change required to generate the active enzyme (*47*). Isozymic differences may be due to sequence differences in the hypervariable loop region and presumably reflect the metabolic flux of the tissues wherein these isozymes are expressed, although confirmation of this role awaits identification of mutations in these loci or development of transgenic animal models.

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