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A Novel Ubiquitin *C*-terminal Hydrolase (UCH-9) from Chick Skeletal Muscle: Its Purification and Characterization

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We have previously shown that chick muscle extracts contained at least 10 different ubiquitin *C*-terminal hydrolases (UCHs). In the present studies, one of the enzymes, called UCH-9, was purified by conventional chromatographic procedures using ^{125}I-labeled ubiquitin-αNH-MHISPPEPESEEEEE-HYC (Ub-PESTc) as a substrate. The purified enzyme behaved as a 27-kDa protein under both denaturing and nondenaturing conditions, suggesting that it consists of a single polypeptide chain. It was maximally active at pHs between 7 and 8.5, but showed little or no activity at pH below 6 and above 10. Like other UCHs, its activity was strongly inhibited by sulfhydryl blocking reagents, such as iodoacetamide, and by Ub-aldehyde. In addition to Ub-PESTc, UCH-9 hydrolyzed Ub-αNH-protein extensions, including Ub-αNH-carboxyl extension protein of 80 amino acids and Ub-αNH-dihydrofolate reductase. However, this enzyme was not capable of generating free Ub from mono-Ub-εNH-protein conjugates and from branched poly-Ub chains that are ligated to proteins through εNH-isopeptide bonds. This enzyme neither could hydrolyze poly-His-tagged di-Ub. These results suggest that UCH-9 may play an important role in production of free Ub and ribosomal proteins from their conjugates.

Ubiquitin (Ub) is a highly conserved 76-amino acid polypeptide. This small protein is involved in a variety of cellular functions, including regulation of intracellular protein breakdown, cell cycle regulation, and stress response (Glotzer et al., 1991; Hochstrasser et al., 1991; Hershko and Ciechanover, 1992; Jentsch, 1992; Wilkinson, 1996). Ub is covalently ligated to target proteins through an isopeptide linkage between the *C*-terminal Gly residue of Ub and the ε-amino group of Lys residue(s) of the proteins. Ubs by themselves or that have already been conjugated to proteins may also be ligated to additional Ub molecules to form branched poly-Ub by the linkage between the ε-amino group of Lys-48 of one Ub and the *C*-terminus of the other. Proteins ligated to multiple units of Ub are degraded by the 26S proteasome (Hershko and Ciechanover, 1992; Jentsch, 1992; Coux et al., 1996; Wilkinson, 1996).

In all eukaryotic cells, Ubs are encoded by two distinct classes of gene, none of which encodes monomeric form of Ub (Ozkaynak et al., 1984; Lund et al., 1985). One is a poly-Ub gene which encodes a polyprotein of up to 100 uninterrupted, tandemly repeated Ubs through peptide bonds between the *C*-terminal Gly and *N*-terminal Met of contiguous Ub molecules. The other encodes a fusion protein, of which a single Ub is linked to a ribosomal protein consisting of 52 or 76-80 amino acids. The transient association of Ub with the ribosomal proteins has been suggested to promote their incorporation into ribosomes (Finley et al., 1989). Therefore, proteolysis at the peptide bonds between Ub and carboxyl extension proteins is required for generation of ribosomal proteins for ribosome biogenesis as well as of free Ubs.

A number of UCHs that release Ub molecules that are conjugated to proteins by αNH-peptide bonds and/or εNH-isopeptide linkages have been identified from different sources. *Saccharomyces cerevisiae* contains at least 5 different UCHs, including yeast ubiquitin *C*-terminal hydrolase-1 (YUH1) and four Ub-specific proteases (UBPs) (Miller et al., 1989; Tobias and Varshavsky, 1991; Baker et al., 1992; Papa and Hochs-

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trasser, 1993). A family of UCHs, named L1, L2, L3, and H2, has been identified from calf thymus (Mayer and Wilkinson, 1989; Wilkinson et al., 1989; Wilkinson et al., 1992). Isopeptidase T in human red blood cells has been purified, and its cDNA has been isolated (Falquet et al., 1995a; Falquet et al., 1995b). In addition, the *fat facets* gene in *Drosophila*, which is required in eye development, and the *DUB-1* gene in murine pro-B cell line, which is specifically induced by IL-3 and involved in growth suppression, have recently been shown to encode a UCH (Huang et al., 1995; Zhu et al., 1996). We also have identified at least 10 different UCHs in chick skeletal muscle using Ub-PESTc (Yoo et al., 1989; Woo et al., 1995; Chung et al., 1996). Since the Tyr residue next to the *C*-terminal Cys can be exclusively radio-iodinated, we could assay the UCH activity by simple measurement of the radioactivity of the peptide portion that is released into acid-soluble products.

Although the number of UCHs identified from various organisms are rapidly growing, only a few of them have been purified and characterized *in vitro*. In the present studies, we have purified a new UCH in chick skeletal muscle, named UCH-9, and shown its biochemical and physicochemical properties.

Materials and Methods

Materials

Ub-PESTc, Ub-αNH-carboxyl extension protein of 80 amino acids (Ub-CEP80), Ub-αNH-dihydrofolate reductase (Ub-DHFR), and poly-His-tagged di-Ub (His-di-Ub) were purified as described previously (Woo et al., 1995). The purified Ub-PESTc was radiolabeled with $Na^{125}I$ using Iodo-Beads (Pierce) (Markwell, 1982). Ub-aldehyde was prepared by borohydride reduction of Ub in the presence of YUH1 as described (Hershko and Rose, 1987).

Assay of UCH activity

Reaction mixtures (0.1 ml) contained a proper amount of the purified UCH-9 or chromatographic fractions and 1 μg of ^{125}I-labeled Ub-PESTc (10^4 cpm/μg) in 100 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 1 mM EDTA, and 5% (v/v) glycerol. After incubating the mixture for various periods at 37℃, the reaction was terminated by addition of bovine serum albumin and trichloroacetic acid to final concentrations of 0.3% (w/v) and 10% (w/v), respectively. The samples were centrifuged, and the resulting supernatants were counted for their radioactivity using a gamma counter. The enzyme activity was expressed as a percentage of ^{125}I-Ub-PESTc hydrolyzed to acid-soluble products.

When assaying for hydrolysis of Ub-αNH-carboxyl extension proteins and His-di-Ub, incubations were performed as above but in the presence of 1 μg of the substrates. After incubation for appropriate periods, the samples were subjected to discontinuous gel electrophoresis (see below). Proteins in the gels were then visualized by staining with Coomassie blue R-250 or by exposing to X-ray films (Fuji) at -70℃.

Preparation of muscle extracts

Chick pectoralis muscle tissues (750 g) were minced and homogenized using a Waring blender in buffer A [25 mM Tris-HCl buffer (pH 7.8) containing 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol] in the presence of 100 mM NaCl. The homogenates were centrifuged at 10,000 × g for 1 h to remove cell debris, and their supernatants were centrifuged again at 100,000 × g for 2 h. The resulting supernatants were titrated with 1 M Tris base to pH 7.8 and referred to as the muscle extracts.

Electrophoresis

Polyacrylamide gel electrophoresis in the presence and absence of SDS and 2-mercaptoethanol was performed as described by Laemmli (1970) or using Tris-Tricine buffer as described by Schäger and Von Jagow (1987). The discontinuous slab gels contained 4, 10, and 16% (w/v) polyacrylamide to improve resolution of small proteins. The sample buffer contained 150 mM Tris-HCl (pH 6.8), 1.5% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue, and 7% glycerol.

Results

Purification of UCH-9

To separate UCH-9 from the other UCHs, extracts (18.3 g protein) were prepared from 750 g of chick skeletal muscle (Woo et al., 1995). The activity of UCH-9 in chromatographic fractions obtained during purification was monitored by determining its ability to hydrolyze ^{125}I-labeled Ub-PESTc. The muscle extracts were applied to a DEAE-Sepharose column (2.5 × 18 cm) equilibrated with buffer A containing 0.1 M NaCl. After washing with the same buffer, the proteins bound to the column were eluted with a linear gradient of 0.1 to 0.3 M NaCl. The active fractions were pooled, dialyzed against buffer A, and loaded onto a heparin-Sepharose column (2.5 × 8 cm) equilibrated with the same buffer. The bound proteins were eluted with a linear gradient of 0 to 0.6 M NaCl, and the active fractions were pooled and dialyzed against buffer A containing 0.1 M NaCl. The dialyzed sample was chromatographed on a Sephacryl S-300 gel filtration column (2.5 × 76 cm) equilibrated with buffer A. The active fractions were pooled, diluted to 2-fold with buffer A containing 2 M ammonium sulfate, and loaded onto a phenyl-Superose column (0.5 × 5 cm). The bound proteins were separated by a reverse gradient of 1.0 to 0 M ammonium sulfate. The active fractions

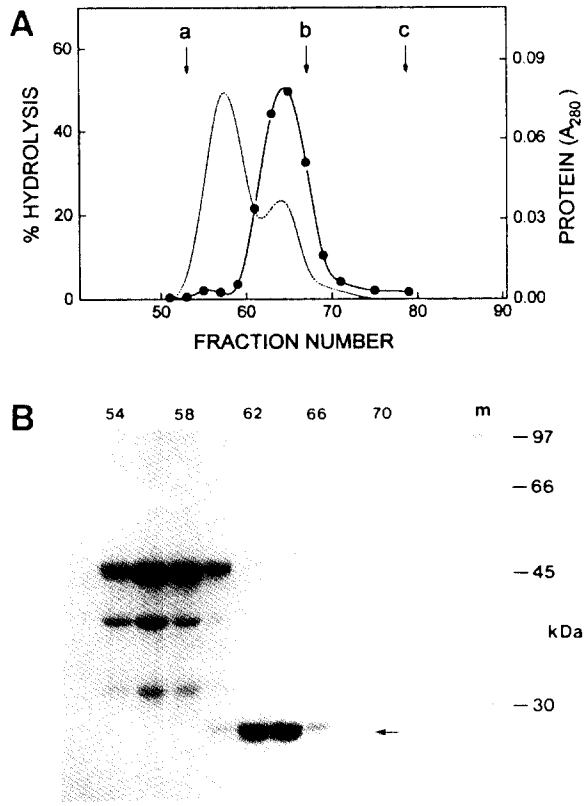

Fig. 1. Separation of UCH-9 on a Superdex-75 column. A, The UCH-9 preparation from the phenyl-Superose chromatography was loaded onto a Superdex-75 column as described in the text. Fractions of 1 ml were collected and aliquots (2 μl) of them were assayed for their ability to hydrolyze ^{125}I-labeled Ub-PESTc (●) as described in Materials and Methods. The size markers used are: a, bovine serum albumin (66 kDa); b, carbonic anhydrase (29 kDa); c, cytochrome c (12.4 kDa). The dotted line indicates the protein profile. B, Aliquots (80 μl) of the same fractions were electrophoresed on a 12% polyacrylamide gel containing SDS and 2-mercaptoethanol. Proteins in the gel were then visualized by staining with Coomassie blue R-250.

were pooled, dialyzed against buffer A containing 0.1 M NaCl, and concentrated to 2 ml using a Centricon (Amicon). The sample was then subjected to gel filtration chromatography on a Superdex-75 column (1.6 × 60 cm).

A single peak of the activity against ^{125}I-labeled Ub-PESTc was eluted in the fractions corresponding to about 27 kDa (Fig. 1A). Upon analysis by polyacrylamide gel electrophoresis under denaturing conditions, the elution pattern of the activity was found to be tightly correlated with the intensity of the 27 kDa band (Fig. 1B). Thus, the active fractions containing only the 27-kDa protein were concentrated as above and kept frozen at -70℃ for further use. Summary of the purification of UCH-9 was shown in Table 1. Since the size of UCH-9 estimated by the gel electrophoresis under denaturing conditions as well as by the gel filtration analysis under nondenaturing conditions is identical, the enzyme appears to comprise a single polypeptide.

Table 1. Summary of purification of UCH-9

Steps	Protein (mg)	Total activity (units[a])	Specific activity (units/mg)	Yield (%)	Fold
Crude extracts	18,304.00	-[b]	-	-	-
DEAE-Sepharose	480.00	-	-	-	-
Heparin-Sepharose	129.00	-	-	-	-
Sephacryl S-300	13.00	41,000	3,162	100	1.0
Phenyl-Superose	1.20	12,600	10,500	38	3.3
Superdex-75	0.17	7,938	46,694	19	14.8

[a] One unit was defined as 1 μg of Ub-PESTc hydrolyzed to acid soluble products per h.
[b] Not determined.

Biochemical properties

We have previously shown that poly-*L*-lysine stimulates the Ub-PESTc-hydrolyzing activity of all UCHs, except UCH-1, that are partially purified from chick skeletal muscle (Woo et al., 1995). To determine whether the activity of the purified UCH-9 is also stimulated by poly-*L*-lysine, the enzyme was incubated with ^{125}I-labeled Ub-PESTc in the presence and absence of the reagent. As shown in Fig. 2A, poly-*L*-lysine increased the hydrolytic rate of ^{125}I-labeled Ub-PESTc. Moreover, its stimulation occurred in a concentration-dependent fashion (Fig. 2B). The enzyme activity was also stimulated by poly-*L*-arginine to a similar extent, but not by other poly-cationic agents, such as spermine, spermidine, and histone (Table 2). Thus, the stimulation of the UCH-9 activity by poly-*L*-lysine appeared not due to a simple charge effect.

We examined the effects of varying pHs on the hydrolysis of ^{125}I-labeled Ub-PESTc by UCH-9 using various buffers. UCH-9 was maximally active at pH near 8, but was inactive at pH below 6 and above 10, whether or not poly-*L*-lysine was present (Fig. 3).

Since all of the UCHs so far been identified are sensitive to inhibition by sulfhydryl blocking agents (Mayer and Wilkinson, 1989; Miller et al., 1989; Tobias and Varshavsky, 1991; Baker et al., 1992; Papa and

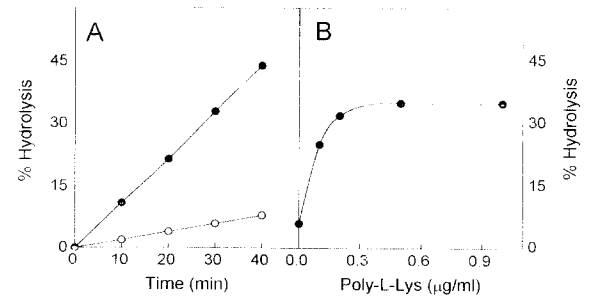

Fig. 2. Effect of poly-*L*-lysine on the hydrolysis of Ub-PESTc by UCH-9. A, Reaction mixtures containing 5 ng of UCH-9 and 1 μg of ^{125}I-labeled Ub-PESTc (1.2×10^4 cpm/g) were incubated in the absence (○) and presence of 1 g/ml of poly-*L*-Lys (●) for various periods at 37℃. B, The enzyme activity was also assayed as above but in the presence of increasing amounts of poly-*L*-lysine. Incubations were performed for 30 min at 37℃.

Table 2. Effect of various polycations on UCH-9

Addition	% Hydrolysis	Relative activity
None	2.7	100
Histone	1.3	48
Spermidine	2.7	100
Spermine	3.4	126
poly-*L*-Arg	21.6	800
poly-*L*-Lys	26.6	985

Hochstrasser, 1993), we also examined the effect of the reagent on the activity of the purified UCH-9 against ^{125}I-labeled Ub-PESTc. As shown in Table 3, UCH-9 was highly sensitive to inhibition by sulfhydryl blocking reagents, such as *N*-ethylmaleimide (NEM) and iodoacetic acid (IAA), suggesting that the enzyme contains a conserved Cys residue for catalysis, similar to other known UCHs (Mayer and Wilkinson, 1989; Miller et al., 1989; Tobias and Varshavsky, 1991; Baker et al., 1992; Papa and Hochstrasser, 1993). Ub-aldehyde, that is known to inhibit the activity of certain mammalian UCHs, including isopeptidase T (Stein et al., 1995; Wilkinson et al., 1995), also strongly inhibited the hydrolysis of ^{125}I-labeled Ub-PESTc by UCH-9. In addition, the activity of UCH-9 was found to be inhibited upon treatment of 0.3 M KCl. On the other hand, inhibitors of serine proteases, such as phenylmethylsulfonyl fluoride (PMSF), or metal chelating agents, such as *o*-phenanthroline, showed little or no effect.

Substrate specificity

To determine the substrate specificity of UCH-9, the purified enzyme was incubated with various Ub-αNH-protein extensions, such as Ub-DHFR, Ub-CEP80, and His-di-Ub. After incubation, the samples were subjected to discontinuous gel electrophoresis under denaturing conditions followed by staining with Coomassie blue R-250. Assays were also performed with Ub-PESTc as a control. As shown in Fig. 4, UCH-9 was capable of generating free Ub from Ub-DHFR and Ub-CEP80. However, it could not hydrolyze His-di-Ub. This enzyme could not generate free Ub from mono-Ub-εNH-protein conjugates or from branched poly-Ub chains that are ligated to proteins through εNH-isopeptide bonds (data not shown). Thus, it appears that UCH-9 specifically hydrolyzes only the Ub-NH-protein extensions, such as Ub-ribosomal protein conjugates (e.g., Ub-CEP52 or Ub-CEP80).

Table 3. Effects of protease inhibitors and salt on UCH-9

Addition	Concentration	% Inhibition
IAA	5 mM	80
NEM	5 mM	97
o-phenanthroline	1 mM	30
PMSF	1 mM	32
Leupeptin	10 μg/ml	5
Ub-aldehyde	25 nM	63
KCl	0.3 M	30

Incubations were performed as in Fig. 2B, but in the absence and presence of the indicated amounts of protease inhibitors and salt.

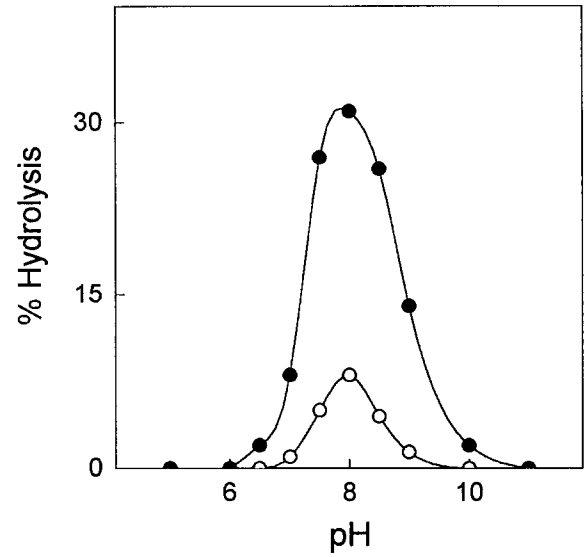

Fig. 3. Effect of pH on the hydrolysis of Ub-PESTc by UCH-9. Effect of pH on the Ub-PESTc-cleaving activity of UCH-9 was determined using various buffers in the absence (○) and presence of 0.4 μg/ml of poly-*L*-Lys (●). The buffers used were: Na-acetate (for pH 4-5), NaH_2PO_4/Na_2HPO_4 (for pH 6-7), Tris-HCl (pH 7.5-8.5), and Na-glycine (pH 9-11). The reaction mixtures were incubated at 37°C for 30 min.

Discussion

In the present study, a new Ub *C*-terminal hydrolase, called UCH-9, in chick skeletal muscle was purified to apparent homogeneity using ^{125}I-labeled Ub-PESTc as a substrate. From 750 g of chick skeletal muscle, we obtained approximately 0.17 mg of the purified enzyme. The purified UCH-9 behaves as a monomer of 27-kDa both under nondenaturing and denaturing conditions.

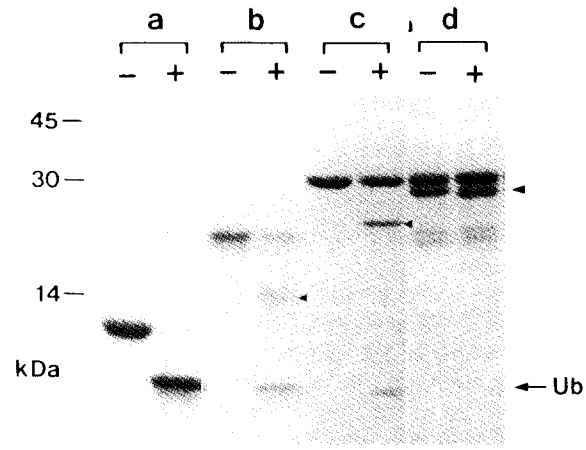

Fig. 4. Hydrolysis of various Ub-αNH-protein extensions by UCH-9. Aliquots (5 μg each) of the purified Ub-PESTc (a), Ub-CEP80 (b), Ub-DHFR (c), and His-di-Ub (d) were incubated for 2 h at 37°C in the absence ("-" lanes) and presence of 0.11 μg of the purified UCH-9 ("+" lanes). After incubation, the samples were subjected to discontinuous gel electrophoresis as described under Materials and Methods. Proteins in the gels were then visualized by staining with Coomassie blue R-250. The small arrowheads in b, c, and d indicate the position where CEP80, DHFR, and His-mono-Ub migrated, respectively. The large arrowhead in d indicates His-di-Ub. The protein band seen just above His-di-Ub (in d) is an unknown protein contaminated during preparation of the substrate.

UCH-9 can hydrolyze Ub-αNH-protein extensions, such as Ub-CEP80 and Ub-DHFR. However, it is not capable of releasing free Ub from His-di-Ub or mono-Ub-ε NH-protein conjugates. Thus, UCH-9 in muscle may play a role in generation of free Ub and the 52- and 80-kDa ribosomal proteins from Ub-ribosomal protein (i.e., Ub-CEP80 and Ub-CEP52) and thus in the ribosomal biogenesis.

Noteworthy is the observation that poly-*L*-lysine dramatically stimulates the Ub-PESTc-cleaving activity of UCH-9. However, the same agent does not exhibit any stimulatory effect on the hydrolysis of Ub-CEP80, Ub-DHFR or His-di-Ub (data not shown). We have previously shown that poly-*L*-lysine stimulates the Ub-PESTc-cleaving activity of all of the UCHs (i.e., UCH-2 to -10) that were partially purified from chick skeletal muscle, except UCH-1. In addition, UCH-1 does not bind to DEAE-column at neutral or slightly alkaline pH (e.g., 7.8), while the others, including UCH-9, strongly interacts with the ion-exchange matrix. These results suggest that the enzymes that are stimulated by poly-*L*-lysine have acidic isoelectric points (pIs) while UCH-1 has a neutral or alkaline pI (Woo et al., 1997). Wilkinson and coworkers have recently reported that the UCHs from yeast, *Drosophila*, and human neuronal and B-cells have acidic pIs (about 5.0) (Falquet et al., 1995; Larsen et al., 1996). Of these enzymes, we have previously shown that the activity of the YUH1 against Ub-PESTc is also markedly stimulated by poly-*L*-lysine (Woo et al., 1995). Therefore, it appears that poly-*L*-lysine may facilitate the interaction between the enzymes having acidic pIs and the Ub-PESTc substrate by neutralizing their negative charges under the assay conditions. However, it remains unclear why the other small poly-cationic reagents, such as spermine or spermidine, are unable to stimulate the Ub-PESTc-cleaving activity of UCH-9 and the other UCHs having acidic pIs.

UCH-9 appears to be distinct from any other known UCHs in a number of criteria: (1) It behaves differently from other chick UCHs on chromatographic columns; (2) Its native molecular mass of 27 kDa is the smallest among the chick UCHs; (3) Its substrate specificity is limited to Ub-αNH-protein extensions, similar to that of UCH-6 but unlike of the other chick UCHs; (4) It is much more sensitive to activation by poly-*L*-Lys compared to the other UCHs whose activities are also stimulated by the reagent (i.e., the concentration of poly-L-Lys required for a half-maximal stimulation for UCH-9 is less than 0.1 μg/ml, while that for other UCHs is at least 1 μg/ml) (Woo et al., 1995; Baek et al., 1997). Studies on the cloning of its cDNA and subsequent sequence analysis should help in clarification of the distinctive nature of the enzyme and ultimately of its physiological function.

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References

Baker RT, Tobias JW, and Varshavsky A (1992) Ubiquitin-specific proteases of *Saccharomyces cerevisiae*: Cloning of *UBP2* and *UBP3*, and functional analysis of the *UBP* gene family. *J Biol Chem* 267: 23364-23375.

Baek SH, Woo SK, Lee JI, Yoo YJ, Cho CM, Tanaka K, Kang MS, and Chung CH (1997) A new deubiquitinating enzyme, ubiquitin *C*-terminal hydrolase-8 in chick skeletal muscle. *Biochem J* (in press).

Bond U and Schlesinger MJ (1986) The chicken ubiquitin gene contains a heat shock promoter and expresses an unstable mRNA in heat-shocked cells. *Mol Cell Biol* 6: 4602-4610.

Chung CH, Woo SK, Lee JI, Park IK, Yoo YJ, Cho CM, Kang MS, and Ha DB (1996) Multiple ubiquitin *C*-terminal hydrolases from chick skeletal muscle. *Adv Exp Med Biol* 389: 203-208.

Coux O, Tanaka K, and Goldberg AL (1996) Structure and functions of the 20S and 26S proteasomes. *Annu Rev Biochem* 65: 801-847.

Falquet L, Paquet N, Fruitger S, Hughes GJ, Hoang-Van K, and Jaton JC (1995a) A human deubiquitinating enzyme with both isopeptidase and peptidase activities *in vitro*. *FEBS Lett* 359: 73-77.

Falquet L, Paquet N, Fruitger S, Hughes GJ, Hoang-Van K, and Jaton JC (1995b) cDNA cloning of a human 100-kDa de-ubiquitinating enzyme: the 100-kDa human de-ubiquitinase belongs to the ubiquitin *C*-terminal hydrolase family 2 (UCH2). *FEBS Lett* 376: 233-237.

Finley D, Bartel B, and Varshavsky A (1989) The tails of ubiquitin precursors are ribosomal proteins whose fusions to ubiquitin facilitates ribosome biogenesis. *Nature* 338: 394-401.

Glotzer M, Murray AW, and Kirschner MW (1991) Cyclin is degraded by the ubiquitin pathway. *Nature* 349:132-138.

Hershko A and Ciechanover A (1992) The ubiquitin system for protein degradation. *Annu Rev Biochem* 61: 761-807.

Hershko A and Rose IA (1987) Ubiquitin-aldehyde: a general inhibitor of ubiquitin-recycling processes. *Proc Natl Acad Sci USA* 84: 1829-1833.

Hochstrasse M, Ellison MJ, Chau V, and Varshavsky A (1991) The short-lived MAT2 transcriptional regulator is ubiquitinated *in vivo*. *Proc Natl Acad Sci USA* 88: 4606-4610.

Huang Y, Baker R, and Fischer-Vize JA (1995) Control of cell fate by a deubiquitinating enzyme encoded by the *fat facets* gene. *Science* 270: 1828-1831.

Jentsch S (1992) The ubiquitin-conjugation system. *Annu Rev Genet* 26: 179-207.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Larsen CN, Price JS, and Wilkinson KD (1996) Substrate binding and catalysis by ubiquitin *C*-terminal hydrolases: Identification of two active site residues. *Biochemistry* 35: 6735-6744.

Lund PK, Moats-Staats BM, Simmons JG, Hoyt E, D'Ercole AJ, Martin F, and van Wyk JJ (1985) Nucleotide sequence analysis of a cDNA encoding human ubiquitin reveals that ubiquitin is synthesized as a precursor. *J Biol Chem* 260: 7609-7613.

Markwell MAK (1982) A new solid-state reagent to iodinate proteins. *Anal Biochem* 125: 427-432.

Mayer AN and Wilkinson KD (1989) Detection, resolution, and nomenclature of multiple ubiquitin carboxyl-terminal esterases from bovine calf thymus. *Biochemistry* 28: 166-172.

Miller HI, Henze, WJ, Ridgway JB, Kuang WJ, Chisholm V, and Liu CC (1989) Cloning and expression of a yeast ubiquitin-protein cleaving activity in *E. coli*. *Biotechnology* 7:

698-704.
Ozkaynak E, Finley D, and Varshavsky A (1984) The yeast ubiquitin gene: Head-to-tail repeats encoding a polyubiquitin precursor protein. *Nature* 312: 663-666.
Papa FR and Hochstrasser M (1993) The yeast *DOA4* gene encodes a deubiquitinating enzyme related to a product of the human *TRE-2* oncogene. *Nature* 366: 313-319.
Schäger H and Von Jagow G (1987) Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166: 368-379.
Stein RL, Chen Z, and Melandri F (1995) Kinetic studies of isopeptidase T: Modulation of peptidase activity by ubiquitin. *Biochemistry* 34: 12616-12623.
Tobias JW and Varshavsky A (1991) Cloning and functional analysis of the ubiquitin-specific protease *UBP1* of *Saccharomyces cerevisiae. J Biol Chem* 266: 12021-12028.
Wilkinson KD, Tashayer VL, O'Connor LB, Larsen CN, Kasperek E, and Pickart CM (1995) Metabolism of the polyubiquitin degradation signal: Structure, Mechanism, and role of isopeptidase T. *Biochemistry* 34: 14535-14546.
Wilkinson KD (1996) Roles of ubiquitination in proteolysis and cellular regulation. *Annu Rev Nut* 15: 161-189.
Wilkinson KD, Deshpande S, and Larsen CN (1992) Comparisons of neuronal (PGP9.5) and non-neuronal ubiquitn *C*-terminal hydrolases. *Biochem Soc Trans* 20: 631-636.
Wilkinson KD, Lee KM, Deshpande S, Duerksen-Hughes P, Boss JM, and Pohl J (1989) The neuron-specific PGP9.5 is a ubiquitin carboxyl-terminal hydrolase. *Science* 246: 670-673.
Woo SK, Lee JI, Park IK, Yoo YJ, Cho CM, Kang MS, Ha DB, Tanaka K, and Chung CH (1995) Multiple ubiquitin *C*-terminal hydrolases from chick skeletal muscle. *J Biol Chem* 270: 18766-18773.
Woo SK, Baek SH, Lee JI, Yoo YJ, Cho CM, Kang MS, and Chung CH (1997) Purification and characterization of a new ubiquitin *C*-terminal hydrolase (UCH-1) with isopeptidase activity from chick skeletal muscle. *J Biochem* 121: 684-690.
Yoo YJ, Rote K, and Rechsteiner M (1989) Synthesis of peptides as cloned ubiquitin extension. *J Biol Chem* 264: 17078-17083.
Zhu Y, Carroll M, Papa FR, Hochstrasser M, and D'Andrea AD (1996) DUB-1, a deubiquitinating enzyme with growth suppressing activity. *Proc Natl Acad Sci USA* 93: 3275-3279.

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