A novel family of ubiquitin-specific proteases in chick skeletal muscle with distinct N- and C-terminal extensions

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We have recently identified a cDNA for a ubiquitin-specific protease (UBP), UBP41, that encodes the smallest functional UBP identified to date, using an *Escherichia coli*-based *in vivo* screening method. In the present study we isolated highly related cDNAs encoding a new family of UBP enzymes, named UBP46, UBP52 and UBP66. These UBPs have virtually identical catalytic domains spanning the sequence of UBP41 between the active-site Cys and the His box (95% identity). However, they possess distinct N- and/or C-terminal extensions. Moreover, they are more closely related to each other than to any other members of the UBP family. Thus these chick UBPs must define a novel family of de-ubiquitinating enzymes and should represent the first example among the UBP family enzymes, whose multiplicity is achieved by variation in their N- and C-terminal extensions. The chick UBPs were expressed in *E. coli*, and purified from the cells to apparent homogeneity using ¹²⁵I-labelled ubiquitin-αNH-

MHISPPEPESEEEEEHYC as a substrate. Each of the purified UBP46, UBP52 and UBP66 enzymes behaved as proteins of similar sizes under both denaturing and non-denaturing conditions, suggesting that all of them consist of a single polypeptide chain. The UBP enzymes cleaved the C-terminus of the ubiquitin moiety in natural and engineered fusions irrespective of their sizes and thus are active against ubiquitin-β-galactosidase as well as a ubiquitin C-terminal extension protein of 80 amino acids. All UBPs except UBP66 released free ubiquitin from poly-His-tagged di-ubiquitin. However, the isopeptidase activity for hydrolysing polyubiquitinated lysozyme conjugates was not detected from these UBPs, which makes these UBPs distinct from UBP41. These results suggest that the chick UBPs may play an important role in production of free ubiquitin from linear polyubiquitin chains and of certain ribosomal proteins from ubiquitin fusion proteins.

INTRODUCTION

Covalent modification of proteins by the 76-residue ubiquitin (Ub) polypeptide is involved in many aspects of protein metabolism and cellular functions, including elimination of abnormal proteins, cell-cycle progression, signal transduction, transcription and antigen presentation [1–3]. The known substrates of this ubiquitination pathway include protein kinase (Mos), cyclins, transcription factors (Matα2, GCN4, c-Jun, p53, and NF-κB), inhibitors of cyclin-dependent kinases and subunits of trimeric G-proteins [4,5]. The post-translational coupling of Ub to other proteins is catalysed by a family of Ub-conjugating enzymes, called E2s [6,7], 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, either by themselves or 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 recognized by the 19 S regulatory complex of the 26 S proteasome. Proteins bound to the 19 S complex are then probably unfolded and translocated into the central cavity of the 20 S proteasome, where they are degraded to small polypeptides in an ATP-dependent fashion [1–5,8].

Ubs are encoded by two distinct classes of genes, neither of which encodes a monomeric form of Ub [9,10]. One is a poly-Ub gene, which encodes a linear polymer of Ub that is linked through peptide bonds between the C-terminal Gly and N-terminal Met of contiguous Ub molecules. The other encodes a fusion protein in which a single Ub is linked to a ribosomal protein consisting of 52 or 76–80 amino acids [11]. Thus generation of free Ub from the linear poly-Ub and Ub fusion proteins and recycling of Ub from the branched poly-Ub ligated to proteins should be essential for Ub-requiring processes, including intracellular protein breakdown of ubiquitinated proteins by the 26 S proteasome.

A large family of genes encoding de-ubiquitinating enzymes has recently been identified from different organisms. *Saccharomyces cerevisiae* (baker's yeast) contains 17 different Ub C-terminal hydrolases (UCHs), including yeast Ub hydrolase 1 (YUH1) and Ub-specific protease (UBP) 1–16 [12–15]. Of these, UBP3 has been shown to regulate silencing by interaction with SIR4, which is required for silencing of transcription at the silent-mating-type loci and at telomers in yeast [16]. Yeast UBP14 enzyme has been recently reported to facilitate proteolysis *in vivo* by preventing unanchored Ub chains from competitively inhibiting polyubiquitinated substrate binding to the 26 S proteasome [17]. A family of UCHs, including L1, L2, L3 and H2,

Abbreviations used: Ub, ubiquitin; UCH, ubiquitin C-terminal hydrolase; YUH1, yeast ubiquitin hydrolase 1; gal, galactosidase; UBP, ubiquitin-specific protease; *ubp*, UBP open reading frame; Ub-PESTc, ubiquitin-αNH-MHISPPEPESEEEEEHYC; CEP80, carboxy extension protein 80; DHFR, dihydrofolate reductase; His-di-Ub, poly-His-tagged di-Ub; Xgal, 5-bromo-4-chloroindol-3-yl β-D-galactoside.

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The nucleotide sequences reported in this paper have been submitted to the Genbank/EMBL/DBJ Nucleotide Sequence Database under the accession numbers AF064248 (*ubp46*), AF064249 (*ubp52*) and AF064250 (*ubp66*).

GAATTCGGCATAGCTCCTAATCCA (-505)
 ACACCTCCTACGGCTCCACCTATGCGCTATGCAGACAGACAAAATCGGCTTCAACGACA (-480)
 GGTACCTCGCTCCCTCCCTCGGTACACACACAGACAGCTGTCCCAACACAGCGGCTTCCGCA (-420)
 GGGCGCGCTCGCTCCCTGTACCCGACTCCAGACTCCGCTCAGCCCGCTTACACCCCGG (-360)
 CACAGCGCGCGGGGGGGGGCTCATCGGGGGCTCGTCTACTCCTTACCGGGATCAGCC (-300)
 CGAGCCCTCTGGCTAGTGTCCACCTTCCACACTTCTCAGCGCTCGCAGAGCGCAGCA (-240)
 ACTCGACCTGGCGCAGGAGTTTTCGGGGCTGCACAGCTCGGATAGCGCCTACGTGGAGA (-180)
 GCCCTAGTGCCTGTACAGGCTGCCACGGCTCCGACTACGCTCCGCGATTACCGGGATG (-120)
 AGAGACCCACTGCACGCTGCCTCCAGCCCTCCGCGAGCGCTCTGTAAATAGTGT (-60)
 ATGGCGGCAGCATGGCCGACCCCGACGCTCAAGGTGGTGCAGGGGCTCGACTGGG 60
 M A A R M A G C P T P R A F R W C R G S T G 20
 CTGGGAACCTCGGCAATACGTGTACATGAATCCATCCTGCAGTGCCTGAGCAACACC 120
 L R N L G N T C Y M N S I L Q C L S N T 40
 AAGGAGCTGGGGATTTACTGCTGCAGAACCACTACCTCGGGACTTCAACAACACAGC 180
 K E L R D Y C L Q N Q Y L R D F N N N S G 60
 CGCATGGCACCCTGCTATGTAGAGTTTGCAAGCTGATCAGCTGTTCTGGACCTCA 240
 R M R T A F M S E F A K L I Q L F W T S 80
 TCCCCCAATGACAGCGTGAGTCCCTCCGAGTTCAAGACGAGATCCAGAGATACGCCCCA 300
 S P N D S V S P S E F K T Q I Q R Y A P 100
 CGCTTTTGTGGATACACAGCAGGATGCACAGGAGTTCCTGCGCTTCTCCTGGATGGG 360
 R F V G Y N Q Q D A Q E F L R F L L D G 120
 CTGCACGCGAGGTGAACCGCTGCTGGTGGCGCGGGCCAACTGCTGACACCTTGGAC 420
 L H G E V N R V L V R P R A N A D T L D 140
 CACTTCCTGATGATGAGAGCGCGCAGATGTGGCGGAGGTACCAGGAGCGGGAGGAC 480
 H F P D D E K S R Q M W R R Y Q E R E D 160
 AGTCCTTGTAGTGAITTTCTTCTGGGAGCTGAAGAGCTCACTGACCTCCAGTGGTGT 540
 S P V S L D F F L G K S S L T Q S C L 180
 GGCTACTGCTCCACAGCCTTTGACCCCTTCTGGKACTCTCCCTGCCATCCCAAGAAA 600
 G Y C S T A F D P F W D L S L P I P K K 200
 GGCTATGGGAAGTGACCTGATGACCTGCTGCCCTCTTCAACAAGAGGACCTTCTG 660
 G Y G E V T L M G T L F T K E D L L 220
 GATGGGATGAGAAACGACCTGCTGCTGCTGCAAGCCAGGACGCGATGCACCAAGAAA 720
 D G D E K P T C C R K A R T R C T K K 240
 TTACAGCATCCAGAGTTTCCCAAGATCCTCCTGCTCCACCTGAAGCGCTTCTCAGAGGCC 780
 F S I Q K F P K I L L L H L K R F S E A 260
 AGGATCAGAGCCAGTCAAGCTACCTTCTGCTGCTTCCACTGAAGGACCTGGACCTG 840
 R I R A S K L T T F V D F P L K D L D L 280
 AGGGAGTTGCTCGCAGAGCTGCAACCCAGCGCTTACAACTCTACGGCGCTCTTAAC 900
 R E F A S Q S C N H A V Y K L Y G V S N 300
 CACTCGGGCACCACCATCGGGGGACCTACCACTGCTCAAGAGGCCATCTCCAGCG 960
 H S G T T M G G G H Y H C L L K R P S P A 320
 GAGTGCACAGCTTCAACGATTCGCGGCTCAGCCCATGTCTCCACCCAGCTGCGCAGC 1020
 E W H S F N D S R V T P M S T H V R S 340
 AGCGATGCTACCTGCTCTTACGGCTGGCCAGCCCATCTCAGCATGTAGCCGGCCC 1080
 S D A Y L L F Y G W P A H P H A C S R P 360
 CCGGGACCCCTCGGAGCTCTTACTGAGTTTGGGCCCCCAAGAACAGAGAAAGCCC 1140
 P G T P R S L L F G F G P P K N S R K P 380
 CGAGACGAGGGCAGGACCCAGGACGCCAGTGCCTCGGGCGCTGGCGTTTGTCTTTT 1200
 R D E G R T Q A A P V P P G R W R L F 400
 ACCCTGGGTAGGTTTCTTTCTTCTTCTGGTGTGAATAAAATACTGAGCAAGGG 1260
 T L G * 403
 CCGTGGGGTGCTGCTCCACCGGGACCTCGGGGACACCGGAGTTGGGTCTTCTTGG 1320
 CATCTCGTACCACTTCGCATCCCGAGGATCCGTGAGCCTCCTCCAGCTCCTCCAAA 1380
 CCTCCTGTTGAAGGATCGCAGCTCTTGGTAGCCTCCATCCACCTCCATCCTCGTCCG 1440
 ATCGGCCCCAGACTCAGCGCCAGGAGGAGGATGATGCTTATGAGTTTCTTTCTATT 1500
 ATCAGCGACTTCTCAGCCCGGACATCCTCCTGGCTGCCAGGACTCAGGCGGGG 1560
 GGGGCTGACCCACTCCTTATACAGCGGTGCCCTTAAACATTTACGGCGCAGGAGGCC 1620
 TTTGGGTGACAGGATGTCCTGCTACGGGGGGTGGGGGGTGGGACACCTTCTTA 1680
 GTAGTGGGCTTCTCCTCCTCTTACACAGGACCCACGGGTCGCCAGAGATGCTCCC 1740
 CTCACCCAGCCCCACTGCTCGTCTCCAGTAGCAAAAGCCAGTGGGCTTTTATGCTT 1800
 TTTCTTAATTTATCTCCTCCCATCCCGGCTGCGCAGGTGCAATGGGGGGGACATG 1860
 CAACCTACCCAGCAACCCAGCCCGAGTCCCAACCCAGGCCCATCTCCTGTTTGGGCA 1920
 CGGGCTGAGCCCCCTGCCCGATACATCCCCAGGGGATCCAGAGGGGGCAGGGG 1980
 CAGAGTGGGTCCGGGCTGGGGCAGCGCTGCAGCTCCGCGAGTGGCTTGTACTGTGT 2040
 AAGCAAGATGGGAGGATGTGTCTGTACAGAGGAGACTCGTGTAGGAATAAATTTAGCT 2100
 GGAACAGAAAAAAGGAATTC 2122

Figure 1 Nucleotide sequence of the *ubp46* cDNA and deduced amino acid sequence of the UBP46 protein

The nucleotides are numbered on the right, beginning at the A of the presumed start codon. The amino acid residues are also numbered on the right. The asterisk indicates the TAG stop codon.

has been identified from bovine calf thymus [18–20]. Isopeptidase T in human red blood cells has been purified [21] and its cDNA has been isolated from human brain library [22]. Moreover, it has been shown that the 19 S regulatory complex of the 26 S proteasome has intrinsic isopeptidase activity [23].

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 interleukin-3 and involved in growth suppression, have been shown to encode UCHs [24,25]. A neuron-specific UCH in *Aplysia* has been reported as an immediate-early gene essential for long-term facilitation [26]. We also have identified at least ten different UCHs in chick skeletal muscle using Ub- α NH-MHISPPPESEEEEEHYC (referred to as Ub-PESTc) [27–29]. Since the Tyr residue next to the C-terminal Cys can be exclusively radioiodinated, we could assay

the UCH activity by a simple measurement of the radioactivity of the peptide portion that is released into acid-soluble products. Of these, UCH-1 [30], UCH-6 [28] and UCH-8 [31] have been purified and their biochemical properties characterized.

By using an *E. coli*-based *in vivo* screening method, we have recently reported isolation of a cDNA for UBP41 [32]. Here we report the identification of a new family of UBPs related in sequence to UBP41 from the chick muscle cDNA library. We also expressed the enzymes in *E. coli*, purified them, and characterized their biochemical properties.

EXPERIMENTAL

Materials

Ub-PESTc, Ub- α NH-carboxy extension protein of 80 amino acids (Ub-CEP80), Ub- α NH-dihydrofolate reductase (Ub-DHFR), and poly-His-tagged di-Ub (His-di-Ub) were purified as described previously [28]. The purified Ub-PESTc was radio-labelled with Na^{125}I using Iodo-Beads (Pierce) [33]. Ub-aldehyde was prepared by borohydride reduction of Ub in the presence of YUH1 as described in [34].

The chick cDNAs in pBluescript II (pBS) were excised out from the chick skeletal-muscle λ ZAPII cDNA library (Stratagene) by following the procedure recommended by the manufacturer. The cDNA plasmids were propagated in *E. coli* JM101 cells that had been transformed with the plasmids pACUb-R- β -galactosidase (pACUb-R- β -gal) or pACUb-M- β -gal [13,32].

Cloning of a family of UBP open reading frames (*ubps*)

The *E. coli* JM101 cells carrying both the chick cDNA plasmids and pACUb-R- β -gal were plated on to agar plates containing 100 $\mu\text{g}/\text{ml}$ ampicillin, 34 $\mu\text{g}/\text{ml}$ chloramphenicol and 40 $\mu\text{g}/\text{ml}$ 5-bromo-4-chloroindol-3-yl β -D-galactoside (Xgal). They were then incubated at 37 °C for 14 h. Among 10^6 transformants, three appeared as white colonies, while the others were stained blue. All the control cells (i.e. transformed with pBluescript II vector only) developed a blue colour. From the three white colonies, plasmids were isolated and again transformed into the *E. coli* JM101 expressing either Ub-Arg- β -gal or Ub-Met- β -gal. These three colonies turned white when transformed with Ub-Arg- β -gal, but not with Ub-Met- β -gal (i.e. became blue), confirming that the white colonies contain the plasmids carrying the chick cDNAs specifying the UBP activity against the Ub- β -gal proteins.

The DNA fragments of the cDNAs generated by restriction endonucleases were subcloned into pBS vector, and sequenced using Sanger dideoxy techniques (TaqTrack sequencing system, Promega) and an automatic sequencer (Applied Biosystems 373A instrument). All sequences were determined on both DNA strands, and all restriction sites used for subcloning were verified as well.

The plasmid that contains an open reading frame encoding a 46 kDa protein had a much longer 5'-untranslated region than the other two plasmids (see below). Therefore it was digested by *Bgl*II in the 5'-untranslated region and *Xho*I in pBS vector, gap-filled by Klenow enzyme, and ligated by T4 DNA ligase. The resulting plasmid was referred to as 'pBS/Ubp46'. The other two plasmids that carry open reading frames encoding 52 kDa and 66 kDa proteins were also isolated and referred to as 'pBS/Ubp52' and 'pBS/Ubp66'.

Expression of UBPs in *E. coli*

The parental *E. coli* JM101 cells were transformed with pBS/Ubp46, pBS/Ubp52, and pBS/Ubp66 and grown to late expo-

AATTCTCTGGTGCATCCAGAAG (-202)
 TTCCCAAGATCCTGGTGTCTCCACTGAAGCGCTTCTCAGAGGCCAGGATACGAGCAGC (-180)
 AAGCTCACCACCTTCGTCACTTCCCACTGAAGAGCTGGACCTGAGGGAGTTTCGCTCG (-120)
 CAGAGCTGCAACACCGCGTTTACAACTCTACGCGCTCTCAACCACTCGGGCACCAC (-60)
 ATGGGGGACACTACACTGCTACTGCAAGAGCCCACTCCAGCGAGTGGCAGACTTC 60
 M G G H Y T A Y C K S P I S S E W H S F 20
 AACGATTCGCGGTCAACCCATGCTCTGCGCAGTGTCCACTCCACACTTCTCAGC 120
 N D S R V T P M S S G H V S T S T L L S 40
 CGTGCGAGGCGCTCAGCCACTCGGACCTGGCGCAGGAGTTTTCGGGGCTGCACAGCTG 180
 R R R A V S H S D L A Q E F S G L H T S 60
 GATAGCGCTACGCAACGCGCCAGTGCCTGTACAGGCTGCCACGCGCTCCGACTAC 240
 D S A Y A P S P S A L Y Q A A T R S D Y 80
 GTCCGCGATTACCTGGAGAGAGCAGCCACTGCAGCTGCCCTCCAGCCCTCCAGCAGC 300
 V R D Y L E K S S H C S L P S P S S 100
 CAGCTCTGTGAAGGGATGATGGTATGGCGGCACGATGAGCCGACCCACGAGCTCC 360
 Q L C E R D D G Y G G T H G P D P T S S 120
 AAGTGGTGCAGGGCTGACTGGGTGGGGAACCTCGGCAATACGTGCTTATGAACTCC 420
 K V V Q G L T G L G L N L G N T C F M N S 140
 ATTCCTGAGTCTGAGCAACAGGAGCTGCGGCTCATGTGAGAGTTTGAAGAAG 480
 I L Q C L S N T K E L R D Y C L Q N Q Y 160
 CTGCGGAGCTCAACAAACAGCGGCTGCGCAGCGGCTCATGTGAGAGTTTGAAGAAG 540
 L R D L N N N S R M R T A L M S E F A K 180
 CTGATCCAGCTGCTGAGCCTCATCCCAATACAGCGTGAAGTCCCTCCGAGTTCAG 600
 L I Q L L W T S S P N H S V S P S E F K 200
 ACGCAGATCCAGAGATACGCCACGCTTTGTGGATCAACAGCAGGATGCACAGGAG 660
 T Q I Q R Y A P R F V G Y N Q Q D A Q E 220
 TTCTCGCGCTTCTCTGGATGGGTGACGCGAGGTGAACGCGTCTGGTGGCGCGG 720
 F L R F L L D G L H G E V N R V L V R P 240
 CGGGCAATGTGACACCTTGGACACCTTCTGATGATGAGAAGACCGCCAGATGTGG 780
 R A N A D T L D L F D D E K S R Q M W 260
 CGGAGTACAGGAGCGGAGGAGCAGTCTGTAGTATCTCTGCTGGCGAGCTGAAG 840
 R R Y Q E R E D S R V S D L F V A Q L K 280
 AGCTCACTGACCTGAGTGTGGGTACTGCTCCAGCTTGTACCCCTTCTGGGAC 900
 S L T C S E C G Y C S T A F D P F W D 300
 CTCTCCCTGCCATCCCAAGAGGCTATGGGAGTGAACCTGATGGAGTCCCTGGCG 960
 L S L P I P K K G Y G E V T L M D C L R 320
 CTCTTACCAAGAGGAGCTTCTGGATGGGATGAGAAACCGAGTGTCTGCTGCGTCA 1020
 L F T K E D V L D G D E K P T C C R C K 340
 GCCAGGACGCGATGACCAAGAAATTCAGCATCCAGAGTTCCTCAAGATCTCTGGTGC 1080
 A R T R C T K K F S I Q K F P K I L V L 360
 CACCTGAAGCGCTCTCAGAGCGAGGATGAGAGCAGCAGTCCACACTTCTGCTCA 1140
 H L K R F S E A R I R A S K L T T F V N 380
 TTCCCACTGAAGGACCTGGACCTGAGGAGTTCGCTCGCAGACTGCAACACAGCGCT 1200
 F P L K D L D L R E F A S Q S C N H A V 400
 TACAACCTCTACGCGCTCTCAACCACTCGGGGCCACCACTGGGGGACACTACACTGCC 1260
 Y N L Y A V S N H S G A T M G G H Y T A 420
 TACTGCAAGAGCGCCATCTCAGCGAGTGGCAGACTTCAACGATTCGCGGTCAACCCC 1320
 Y C K S P I S S E W H S F N D S R V T P 440
 ATGTCTCAGCAGCTGCGCCACGAGCGTCTGCTGCTCTCTACGACTGCGGACG 1400
 M S S S H V P T S T D A Y L F Y D L A S 460
 CCATCTCAGCGATGTAGCCCCCGGTGGGACCTCGGAGCCTCTACCTGATTTGGG 1440
 P S S R M * 465
 CCCCCAAGAACAGCAGAAAGCCCCGAGACGAGGCGAGGACCCAGGAGCCCCAGTGTCT 1500
 CGGGGCGCTGGCGTTTGTCTTTTAAACCTGGGGTAGGTTTCTTTTCTCTTCTGCTG 1560
 TGTAAATAAAATCTAGCAAGGGCGTGGGGCTGCTCTCCACCGGAGCTCGGGGA 1620
 CACCCGAGTGGTCTCTTCTGGCATCTCGTACCACTCTGCCATCCCCGAGGATCCCT 1680
 GAGCTCTCTCCAGCTCTCTCAAACTCTCTGGTGAAGGATCGCAGCTCTCGTTCGCTC 1740
 CATCCCACTCCATCTCTCTTCCGATCGGCGCAGACTCAGGCGCAGGAGGAGATGAT 1800
 GCTTCTCAGTGTCTTCTTATTTATCAGCACTTCTCTCAGCCCGGACCATCCCTCTG 1860
 GCTGCCAGGAGCTCAGCGCGGGGCGCTGACCCACTCTTATACAGCGGTGCTTAA 1920
 ACCATTTCTGGGCGCAGGAGCCCTCTGGGTGCAACAGGATGCTCTCTACGCGGTTT 1980
 GGGGGTGGCGAGCACTTCTTAGTAGTGGGGCTTCTCTCCCTCTTACCAGGAGCCCC 2040
 ATGGGTCGCCAGAGATGCTCCCTCAGCAGCGCCACTGCTCCGCTCTCAGCTAGCAA 2100
 AGCCAGTGGCTTTTATGCTTTTCTTAATTTATCTCTCTCCCATCCGCGCTGCC 2160
 GAGGTGCAATGGGGGGGACATGCACTGACCCAGCAACCCAGCCCGCTGCAACCC 2220
 AGCCGCTCTCTGTTTGGGACCGGTGAACCCCAATTC 2261

Figure 2 Nucleotide sequence of the *ubp52* cDNA and deduced amino acid sequence of the UB52 protein

nential phase in Luria broth containing ampicillin at 37 °C. The cultures were then treated with isopropyl thio- β -D-galactoside to 2 mM and further incubated for the next 3 h. After incubation, the cells were collected, resuspended in buffer A [25 mM Tris/HCl buffer, pH 7.8, containing 5 mM 2-mercaptoethanol, 1 mM EDTA and 10% (v/v) glycerol] and disrupted in a French press at 96.6 MPa (14000 lbf/in²). The cell suspension was centrifuged at 100000 *g* for 2 h, and the resulting supernatants were referred to as 'crude extracts'.

In vitro assay for UBP activity

For the *in vitro* assays, ¹²⁵I-labelled Ub-PESTc was used as a substrate [28]. Reaction mixtures (0.1 ml) contained a suitable amount of the enzyme preparations and 10–30 μ g of ¹²⁵I-labelled Ub-PESTc in 100 mM Tris/HCl (pH 7.8)/1 mM dithiothreitol/1 mM EDTA/5% (v/v) glycerol. After incubating the mixtures for appropriate periods, the reaction was terminated by adding 50 μ l of 40% (w/v) trichloroacetic acid and 50 μ l of 1.2% (w/v)

GAATTCGCGAGTCCGCC (-17)
 ATGGAGCCCCCGCGCTCCCCGACCTTCAAGAGGAGTGGCGGCCGACCTCCATCCGCG 60
 M E P P A L P D L H K E L R P R T S M P 20
 GGCTCCCTGCTGGTCTCCACTTTCGTGGGCTCGTCTCAACAAGACCAAGAGCTCCAAG 120
 G S L L V S T F V G L V L N K T K S S K 40
 GTGGTGCAGGGGCTGACTGGGCTGCGGAACCTCGGCAATAGTGGTCTCATGAATCCATC 180
 V V Q G L T G L R N L G N T C F M N S I 60
 CTGCACTGCTGAGCAACACCAAGGAGTGGGGATTAAGTCTGCTGAGAGTCCAGAGTCTG 240
 L Q C L S N T K E L R D Y C L Q N Q Y L 80
 CGGGACCTCAACAACACAGCGCATGCGCAGCCCGCTCATGTGAGAGTTTGAAGAAGCTG 300
 R D L N N N S R M R T P L M S E F A K L 100
 ATCCAGCTGCTTGGACCTCATCCCCAATGACAGCGTGAAGTCCGAGTTCAAGAGC 360
 I Q L L W T S S P N D S V S P S E F K T 120
 CAGATCCAGAGATACGCCCCAGCTTTGTGGATACAAACAGCAGGATGACAGCAGGATTC 420
 Q I Q R Y A P R F V G Y N Q Q D A Q E F 140
 CTGCGCTTCTCTGGATGGGCTGACGCGAGGTGAACCGCTGCTGGTGGCGCGCGG 480
 L R F L L D G L H G E V N R V L V R P 160
 GCGAATCTGACACTTGGACACCTCCCTGATGATGAGAAGCGCGCAGATGTGGCGG 540
 A N A D T L D H L P D D E K S R Q M W R 180
 AGGTACAGGAGCGGAGGACAGTCTGTAGTATCTTCTGTTGGCGAGCTGAAGAGC 600
 R Y Q E R E D S R V S D L F V A Q L K S 200
 TCAGTACCTGAGTGTGCTGCTGCTCCACGCTTTGACCCCTTCTGGGAGCTC 660
 S L T C S E C G Y C S T A F D P F W D 220
 TCCCTGCCATCCCCAAGAAAGGCTATGGGAGTGAACCTGATGAGTGTCTGCTGCGCTC 720
 S L P I P K K G Y G E V T L M D C L R N F 240
 TTCAACAAGAGGAGCTTCTGGATGGGATGAGAAACCGAGTGTCTGCTGCGTCAAGAG 780
 F T K E D V L D G D E K P T C C R C K V A 260
 AGGAGCGGATGACCAAGAAATTCAGATCCAGAGATTCCTCCAGATCTGGTGTCTCAC 840
 R T R C T K K F S I Q K F P K I L V L H 280
 CTGAAGCGCTTCTCAGAGGCGAGGATACGAGCAGCAGCTCACCACCTTCTGCTCAACTC 900
 L K R F S E A R I R A S K L T T F V N 300
 CCACTGAAGGAGCTGAGCTGAGGAGTTCGCTGCGCAGCTGCAACAGCGCGTTCAT 960
 P L K D L D L R E F A S Q S C N H A V Y 320
 AACCTTACCGCGTCTGTAACCACTCGGCGACCACTGGGGGACACTACACTGCTCAT 1020
 N L Y A V A N H S G T T M G G H Y T A Y 340
 TGCAAGGAGCCCATCTCCAGGAGTGGCAGCTTCAACGATTCGCGCTCACCCTCAT 1080
 C K E P H L Q E W H S F N D S R V T P M 360
 TCTCCAGGAGCTGCGCAGCGATGCTCTCTCTTCTACAGACTGGCCAGCCCA 1140
 S S S H V R S S D A Y L L F Y E L A S P 380
 TCTTCAGCATATACCGGGCCCCCGGACCCCTCGGAGCTCTCTACCTGGATTTGGGCG 1200
 S S R M Y P A P G H P S E P P T W I W A 400
 CCCCCAAGACAGCAGAAAGCCCCGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG 1260
 P Q E Q Q K A P R R G Q D P G S P S A S 420
 GGGCGCTGGCGTTTGTCTTTTACCCTGGGAGTGTCTCTTTTCTCTTCTGCTGT 1320
 G P L A F A F Y P W G R F F F S F W C 440
 GTAATAAAATACTGAGCAAGGGCGTGGGGCTGCTCTCCACCGGAGCTCGGGGAC 1380
 V I K I L S K G R G A A C S H R D L D G 460
 ACCCGAGTGGGTCTCTTCTGGCATCTCGTACCACTTGCATCCCGGAGGATCCG 1440
 T R S W V S S G I S V P L C H P R G S V 480
 AGCCTCTCCAGCTCTCCAAACCTCTGTGAGGATGCGAGTCTTCGATGAGCTCC 1500
 S L L P A P P P N L L V K D R S S S V A S 500
 ATCCCACTCCATCTGTTCCGATCGGCGCCAGACTCAGGCGAGGAGGAGGATG 1560
 I P P S S F R S A P D S R P G G R M M 520
 CTTCAGGAGTCTTCTTATTTATCAGGACTTCTCTCAGCCCGGAGCATCTCTGCTG 1620
 L H G V S F Y L S R T S L S P D H P S W 540
 CTGCCAGGAGCTCAGGCGGGGGGGCTGACCCACTCTTATACAGCGGTGCTTAA 1680
 L P R D S G R G G L T H L I R R C L K 560
 PATTTCGGGGCGCAGGAGGCGCTTGGGGTGCACAGGATGTCCCTCTACCCGGGGGTT 1740
 C F R T A G G G P W G A Q G C P S Y P G V 580
 GGGGGTGGGAGCACTTCTAGTAGTGGGGCTTCTCTCCCTCTTACACAGAGCCCC 1800
 G W A A P F * 587
 ACGGGGTGCCAGAGATGCTCCCTCACCAGCCCCACTGCTCGTCTCCAGTAGCAA 1860
 ACGCACTGGCCCTTTTAGCCTTTTCTTAATTTATCTCTCCCATCCGCGCTGCC 1920
 GAGGTGCAATGGGGGGGACATGCAACTGACCCAGCAGACCCAGCCAGTCCCAACC 1980
 AGCGCCATCTGCTTTGGGACGCGGCTGAGCGCCCTGCGCCCAATCATATCCCAAGG 2040
 GCATCCAGAGGGGCTGGGGGTAGAGGTGGTCCCGGCTGGGCGAGCGCTGACGCTG 2100
 GCAGTGGCTTCTTACTGTGTAAGCAAGATGGCGAGGATGTGCTCTGTAAGGAGAC 2160
 TCGTGTAGGAATAAATTTAGCTGGAACAGAAAGGAATTC 2202

Figure 3 Nucleotide sequence of the *ubp66* cDNA and deduced amino acid sequence of the UB66 protein

BSA. The samples were centrifuged and the resulting supernatants were counted for their radioactivities using a γ -radiation counter. The enzyme activity was expressed as a percentage of ¹²⁵I-labelled Ub-PESTc hydrolysed to acid-soluble products. When assaying the hydrolysis of Ub- α NH-carboxy extension proteins and His-di-Ub, incubations were performed as described above, but in the presence of 5 μ g of the substrate. After incubation for appropriate periods, the samples were subjected to disc electrophoresis (see below). Proteins in the gels were then revealed by staining with Coomassie Blue R-250.

Electrophoresis

2-Mercaptoethanol/SDS/PAGE was performed as described by Laemmli [35] or using Tris/Tricine buffer as described by Schägger and von Jagow [36]. 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

Cloning of *ubps*

By using an *E. coli*-based *in vivo* screening method [32] we isolated three cDNAs expressing the de-ubiquitinating activity from chick muscle cDNA library. The open reading frames specifying the de-ubiquitinating activity were identified by sub-cloning and nucleotide sequencing of the cDNAs and named as *ubp46*, *ubp52*, and *ubp66*, since these encode proteins of 403 residues (46316 Da), 465 residues (52425 Da), and 587 residues (65768 Da) respectively (Figures 1, 2 and 3). From the deduced amino acid sequence the pI values for UBP46, UBP52, and UBP66 were estimated to be 9.46, 8.39 and 9.45.

These UBPs have virtually identical catalytic domains to those of UBP41, spanning the sequence between the active-site Cys and the His box (95 % identity). However, the enzymes exhibit distinct N- and/or C-terminal extensions (Figure 4A). Table 1 shows the percentage identity of the whole protein sequences

Table 1 Protein sequence comparison of a new family of UBPs

Identity in the whole protein sequences among the chick UBPs was expressed as percentage. The sequence comparison was performed using ClustalW program (<http://www2.ebi.ac.uk/service/stmp>).

	UBP41	UBP46	UBP52	UBP66
UBP41	100			
UBP46	88	100		
UBP52	91	74	100	
UBP66	90	77	71	100

among the chick UBPs upon analysis using the ClustalW program. In addition, sequence alignment demonstrated the similarity of the UBPs with several other de-ubiquitinating enzymes, including human tre-2 [37], mouse DUB-1 [25] and yeast Doa4 [15]. However, the sequence similarity was largely restricted to the conserved Cys, His and Asp domains that are characteristic of the UBP family (Figure 4B). Thus the chick

A

B

Cys Domain					Asp Domain				
			▼				▼		
UBP41	8	TPRSSKVVQGLTGLRNLGNT	CFMNSILQCLS	38	107	QQ-DAQEFL		114	
UBP46	8	TPRAPRWCRGSTGLRNLGNT	CYMNSILQCLS	38	107	QQ-DAQEFL		114	
UBP52	116	DPTSSKVVQGLTGLGNLGNT	CFMNSILQCLS	146	215	QQ-DAQEFL		222	
UBP66	35	KTKSSKVVQGLTGLRNLGNT	CFMNSILQCLS	65	134	QQ-DAQEFL		141	
Doa 4	551	TSSHNYDLDFAVGLENLGNS	CYMNCIIQCIL	581	656	QQ-DCQEF		663	
Dub-1	40	SLSWESPQGPGLQNTGNS	CYLNAALQCLT	70	130	QQ-DAFEFL		138	
Tre-2	204	DRQKVPTEKGATGLSNLGN	TCFMNSSIQCVS	234	303	QQ-DSQELL		310	
His Domain									
		▼	▼						
UBP41	293	YNLYAVSNHSGT	TMG-GGHTAYCKSPISSEWHSFNDSRVTPMSSSHVRS-SDAYLLFYELASPS	354					
UBP46	293	YKLYGVSNHSGT	TMG-GGHTAYCKSPISSEWHSFNDSRVTPMSSSHVRS-SDAYLLFYGWPAPHP	354					
UBP52	401	YNLYAVSNHSGAT	TMG-GGHTAYCKSPISSEWHSFNDSRVTPMSSSHVPT-SDAYLLFYDLASPS	462					
UBP66	320	YNLYAVANHSGT	TMG-GGHTAYCKEPHLQEWHSFNDSRVTPMSSSHVRS-SDAYLLFYELASPS	381					
Doa 4	864	YELYGVACHFGT	LY-GGHTAYVKKGLKKGLYFDDTKYKPVKNKADAINSNAYVLFYHRVYGV	926					
Dub-1	290	YALYAVLVHDGAT	SHSGHYFCCVKAG-HGKWKMDDTKVTRC-DVTSVLNENAYNLFYVQQANL	351					
Tre-2	995	YNLYAISC	HSGILS-GGHTAYAKNP-NCKWYCYNDSSCEEL-HPDEIDTDSAYILFYEQQID	1055					

Figure 4 Sequence comparisons among UBP family

(A) Schematic alignment of UBP41, UBP46, UBP52 and UBP66. The sequences of UBPs are drawn to scale and the first and last residues of each sequence are indicated. White, black and stippled boxes denote regions of sequence similarity containing the conserved Cys, Asp and His residues respectively. (B) Sequence alignment around the conserved Cys, Asp and His residues among UBP family. Sequence similarity between chick UBPs ([32]; the present study), human tre-2 [37], murine DUB-1 [25] and yeast Doa4 [15] is shown.

Table 2 Summary of purification of UBPs

Hydrolysis of ^{125}I -labelled Ub-PESTc was assayed by incubation of 30 μg of the substrate with aliquots of the proteins obtained from each purification step for 15 min at 37 °C. One unit was defined as 1 mg of Ub-PESTc hydrolysed to acid-soluble products/h.

Step	Protein (mg)			Total activity (units)			Specific activity (units/mg)		
	UBP46	UBP52	UBP66	UBP46	UBP52	UBP66	UBP46	UBP52	UBP66
Crude extract	8100	9408	11 610	1140	2822	1161	0.14	0.3	0.1
DEAE-Sepharose	1760	1680	1703	744	2016	596	0.42	1.2	0.35
S-Sepharose	12	10	18	48	140	53	4	14	3
Phenyl-Sepharose	2.9	2.1	5	20	59	25	7	28	5
Phosphocellulose	0.17	0.13	0.22	4.8	8.6	4.1	28	65	18

UBPs, including UBP41, UBP46, UBP52, and UBP66, should form a new family of de-ubiquitinating enzymes.

Purification of UBPs

In order to purify the UBPs, crude extracts (11 g protein) were prepared from 120 g of *E. coli* cells that had been transformed with pBS/Ub46, pBS/Ub52 or pBS/Ub66. The activities of the enzymes in chromatographic fractions obtained during purification were monitored by determining their abilities to hydrolyse ^{125}I -labelled Ub-PESTc. Each extract was applied to a DEAE-Sepharose column (2.5 cm $\times$ 19 cm) equilibrated with buffer A, pH 7.8. The DEAE flow-through fractions were pooled and loaded on to an S-Sepharose column (2.5 cm $\times$ 19 cm) equilibrated with the same buffer. After washing the column, proteins bound to the column were eluted with a linear gradient of 0.1–0.4 M NaCl. Fractions with high activity were pooled, mixed with the same volume of buffer A containing 2.4 M $(\text{NH}_4)_2\text{SO}_4$, and applied to a phenyl-Sepharose column (1 cm $\times$ 4 cm). After washing with buffer A containing 1.2 M $(\text{NH}_4)_2\text{SO}_4$, proteins bound to the column were eluted with a reverse gradient of $(\text{NH}_4)_2\text{SO}_4$ from 1.2 M to 0 M. The active fractions were pooled, dialysed against buffer B (20 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4/5$ mM 2-mercaptoethanol/1 mM EDTA/10 % glycerol, pH 7.8) and loaded on to a phosphocellulose column (1 cm $\times$ 4 cm). After washing with the same buffer, proteins bound to the column were eluted with a linear gradient of 20–400 mM phosphate. The peaks of UBP46, UBP52 and UBP66 were eluted from the columns at about 0.16, 0.11 and 0.22 M respectively, and their protein profiles totally superimposed with their enzyme activities (results not shown). Fractions with high Ub-PESTc-degrading activity were pooled, dialysed against buffer A, concentrated by ultrafiltration, and kept frozen at -70 °C until use. A summary of the purification of the UBP enzymes is shown in Table 2.

Isolated UBP46, UBP52 and UBP66 ran as single bands of 44, 58 and 66 kDa respectively on 2-mercaptoethanol/SDS/PAGE, indicating that they were purified to apparent homogeneity (Figure 5). In addition, these enzymes behaved as 47, 60 and 67 kDa proteins when chromatographed on a Superose-12 column (1 cm $\times$ 30 cm) equilibrated with buffer A containing 0.1 M NaCl (results not shown). Since the enzymes were expressed as fusion proteins and hence had extra amino acids at their N-termini, they have sizes similar to, or slightly larger than, those predicted from their open reading frames under both non-denaturing and denaturing conditions. The results indicate that UBP46, UBP52 and UBP66 each consists of a single polypeptide.

Figure 5 Estimation of the sizes of UBP46, UBP52 and UBP66

The purified enzymes (each 5 μg) were electrophoresed on a 11 % (w/v)-polyacrylamide slab gel containing SDS and 2-mercaptoethanol as described by Laemmli [35]. Proteins in the gel were then stained with Coomassie Blue R-250. Lane m, the size markers; lane a, UBP46; lane b, UBP52; lane c, UBP66.

Biochemical properties

To compare the specific activities of UBP41, UBP46, UBP52 and UBP66 against ^{125}I -labelled Ub-PESTc, the same amounts of the purified enzymes were incubated for various periods at 37 °C. Figure 6 shows that the activities of the enzymes increase linearly with time for at least 30 min and that UBP52 hydrolyses the substrate approx. 50 % as well as UBP41, but 2–4-fold more rapidly than UBP46 and UBP66. We also examined the effects of thiol-group-blocking agents, such as *N*-ethylmaleimide and Ub-aldehyde, both of which are known to inhibit the activities of all UCHs and UBPs so far identified [37,38]. *N*-Ethylmaleimide at 1 mM abolished the activities of UBP46, UBP52 and UBP66 against Ub-PESTc. Ub-aldehyde at 1 μM also completely blocked all of the enzyme activities. However, metal-chelating agents, including *o*-phenanthroline (1 mM), or the inhibitors of serine proteases (such as PMSF at 1 mM), showed little or no effect (results not shown). The enzyme activities were neither influenced by poly-(L-Lys) (results not shown), which is known to stimulate dramatically the Ub-PESTc-degrading activities of YUH1 and most UCHs identified in chick skeletal muscle [28].

Figure 6 Hydrolysis of ^{125}I -labelled Ub-PESTc by the purified UBP-family enzymes

Reaction mixtures contained 0.2 μg of the purified UBP41 (○), UBP46 (■), UBP52 (●), or UBP66 (▲) and 30 μg of ^{125}I -labelled Ub-PESTc $[(0.5-1) \times 10^3 \text{ c.p.m.}/\mu\text{g}]$ in 100 mM Tris/HCl (pH 7.8)/1 mM EDTA/1 mM dithiothreitol/5% glycerol. The mixtures were incubated for various periods at 37 °C.

Substrate specificity

To determine the substrate specificity of the UBPs, the purified enzymes were incubated with various Ub- αNH -protein extensions, including Ub-DHFR, Ub-CEP80, and His-di-Ub. After

incubation, the samples were subjected to disc 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 Figure 7(A), UBP46 and UBP52 were capable of generating free Ub from all of the substrates tested. UBP66 could also hydrolyse Ub-CEP80 and Ub-DHFR, but not His-di-Ub. In order to confirm whether Ub-Met- β -gal can also be hydrolysed *in vitro* by the purified UBPs, the enzymes were incubated with the extracts of *E. coli* JM101 cells transformed with pACUb-M- β -gal and then with anti- β -gal antibody for immunoblot analysis. Figure 7(B) shows that all of the purified enzymes can release β -gal from Ub-Met- β -gal expressed in the *E. coli* extracts.

In our previous study, the purified UBP41 could also act on poly-Ub- ϵNH -lysozyme conjugates to generate mono-ubiquitinated forms of 24 kDa, although the latter molecules were not further degraded to free Ub and lysozyme. To determine whether purified UBP46, UBP52 and UBP66 can also hydrolyse branched poly-Ub chains conjugated to proteins, ^{125}I -labelled poly-Ub- ϵNH -lysozyme conjugates were incubated with the purified enzymes. Surprisingly, these UBPs could not hydrolyse poly-Ub-lysozyme conjugates (results not shown), despite the strong similarity in their sequence with UBP41. Thus, these UBPs seem to have a different substrate specificity, perhaps due to their distinct sequences in the N- and/or C-terminal region.

DISCUSSION

In our previous studies we have cloned a cDNA that encodes a 41 kDa UBP in chick skeletal muscle using an *E. coli*-based *in vivo* screening method [32]. The 357-residue protein has no significant sequence similarity to previously isolated yeast or mammalian UBPs, except for three short regions containing Cys, His, and Asp domains [15]. In addition to the catalytic domains, many UBP family enzymes have N- and/or C-terminal extensions, which have been suggested to control substrate specificity, protein-protein interactions and localization. Interestingly,

Figure 7 Hydrolysis of various Ub- αNH -protein extensions and Ub-Met- β -gal by UBP-family enzymes

(A) Aliquots (5 μg each) of the purified Ub-CEP80, Ub-DHFR and His-di-Ub were incubated alone (lanes a) or with 0.5 μg of the purified UBP46 (lanes b), UBP52 (lanes c), or UBP66 (lanes d) for 2 h at 37 °C. After incubation, the samples were subjected to disc electrophoresis under denaturing conditions as described in the Experimental section. Proteins in the gels were then stained with Coomassie Blue R-250. The arrowhead indicates His-di-Ub. The protein band seen just above His-di-Ub is an unknown protein contaminated during preparation of the substrate. Note that the band intensity of the products (indicated by the dots) from Ub-CEP80 and His-di-Ub is much lower than that from Ub-DHFR an unknown reason(s). (B) The extracts (50 μg) obtained from *E. coli* JM101 cells expressing Ub-Met- β -gal were incubated as described above. After incubation, the samples were electrophoresed on an 8% slab gel and then subjected to immunoblot analysis using anti- β -gal antibody.

UBP41 has neither the N- nor the C-terminal extension, making it the smallest functional UBP identified to date.

In the present study, using the same screening protocol, we have isolated, from chick skeletal muscle, three new UBP enzymes (UBP46, UBP52 and UBP66) that have catalytic domains nearly identical with that of UBP41 but which have distinct N- and/or C-terminal extensions. These chick enzymes have not shown any sequence similarity to 16 yeast UBPs. Thus it is likely that these chick UBP enzymes are unique to multicellular organisms. In addition, no significant sequence similarity between the extensions of the three chick enzymes and known sequences of other UBPs was found on database searching. Furthermore, the N- or C-terminal extensions of the chick UBPs show little sequence similarity among each other and differ in size. Thus the chick UBPs, including UBP41, UBP46, UBP52 and UBP66, must represent a novel family of de-ubiquitinating enzymes.

All of the purified UBP-family enzymes appear to be capable of releasing free Ub from linear Ub conjugates, regardless of the size of the conjugates. Thus these new UBP-family enzymes appear to play a role in production of free Ub molecules from Ub fusion proteins, such as Ub-CEP52 and Ub-CEP80. However, it is noteworthy that only the UBP66 enzyme, having both N- and C-terminal extensions, is unable to hydrolyse His-di-Ub. Moreover, none of the newly isolated UBPs can generate free Ub from branched poly-Ub chains that are ligated to lysozyme molecules, unlike UBP41, which lacks both N- and C-terminal extensions. In addition, the specific activities of the four UBP-family enzymes against Ub-PESTc are also different. For example, UBP41 hydrolyses Ub-PESTc at a rate at least 10-fold higher than that of UBP66, which has both N- and C-terminal extensions. Therefore the N- and/or C-terminal extensions may be involved in regulation of the enzyme activity.

Ub-conjugating enzymes (E2s, UBCs) have a highly conserved region of about 150 amino acid residues, called the 'UBC domain', which harbours the Cys residue required for enzyme-Ub thioester formation. Some E2 enzymes possess additional C-terminal extensions that carry out specialized functions such as substrate specificity and intracellular localization of the enzymes. For example, the C-terminal extensions of yeast UBC2 and UBC3 enzymes contribute to their substrate specificity [39–42], and that of the UBC6 enzyme mediates intracellular localization [43]. Thus, the N- and/or C-terminal extensions of the chick UBP-family enzymes may also be involved in substrate specificity and intracellular localization.

Recently, Jentsch and co-workers have identified a new family of E2 enzymes, called UbcD2, UbcM2, UbcM3, and UbcH8 from higher eukaryotes, including *Drosophila*, mouse, and man [44]. Analogous to the chick UBP enzymes, these four variants of the E2 enzymes have virtually identical UBC domains (94% identity), but differ in their N-terminal extensions. Moreover, D'Andrea and co-workers have reported a family of cytokine-inducible UBP enzymes in mouse, called DUB-1 and DUB-2, which are highly similar throughout their amino acid sequences except for a hypervariable region at their C-termini [25,45]. Thus it is interesting that the structural organization, comprising of the same catalytic domain and varied N- and/or C-terminal extensions, appears to be common for both the UBP and UBC families, and may be evolutionarily used for generation of multiplicity of the enzymes, possibly for their diverse cellular functions which are currently unknown.

Previously we have identified at least ten different Ub C-terminal hydrolase (UCH) activities from the extracts of chick skeletal muscle and have tentatively named them UCH-1–UCH-10 [28]. Of these, UCH-1, UCH-6 and UCH-8 have been purified and their biochemical properties have been characterized [28–31].

UBP46, UBP52 and UBP66 appear to be distinct from the previously isolated UCHs. The activities of UBPs are not at all influenced by treatment with poly-(L-Lys), unlike the chick UCHs, except UCH-1, whose activities are dramatically stimulated by the polycationic agent. In addition, UBPs, but not UCH-1, are capable of converting the Ub- β -gal proteins into Ub and β -gal *in vitro*, although all are active against Ub extension proteins with relatively low molecular masses, such as Ub-CEP80 and Ub-DHFR. Thus UBP46, UBP52 and UBP66 appear to be distinct from the previously identified chick UCHs. The presence of such a large number of UCH/UBP enzymes in eukaryotes, such as in chick skeletal muscle, is not required merely to generate free Ub molecules from their precursors, but is indicative of other regulatory roles in the cellular processes that require Ub molecules.

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