Tissue-specificity, functional characterization and subcellular localization of a rat ubiquitin-specific processing protease, UBP109, whose mRNA expression is developmentally regulated

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INTRODUCTION

A cDNA encoding an ubiquitin-specific processing protease, UBP109, in rat skeletal muscle was cloned and its product was characterized. Northern analysis revealed that UBP109 mRNA is highly expressed in testis and spleen, compared with other tissues. Furthermore, in situ hybridization showed that the level of UBP109 mRNA in liver, spinal cord and brain dramatically changed during embryonic development, indicating that the expression of UBP109 mRNA is developmentally regulated. UBP109 was expressed in Escherichia coli and purified to apparent homogeneity using a 125I-labelled ubiquitin–peptide fusion as a substrate. The purified enzyme cleaved at the C-terminus of the ubiquitin moiety in natural and engineered fusions irrespective of their sizes. UBP109 also released free ubiquitin from poly-His-tagged penta- ubiquitin. Moreover, it released free ubiquitin from poly-ubiquitinated protein conjugates of rabbit reticulocytes. In addition, UBP109 localized to both the cytoplasm and the nucleus and, among three putative nuclear localization sequences, only the one located near the C-terminus is responsible for nuclear localization. These results suggest that UBP109 may play an important role in generation of free ubiquitin from its precursors and its recycling from poly-ubiquitinated protein conjugates, and hence in regulation of ubiquitin-mediated cellular processes, particularly related to embryonic development.

Key words: de-ubiquitinating enzyme, embryonic development, hUSP15, nuclear localization, UBP109.

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 [1,2]. The known substrates of this ubiquitination pathway include protein kinase (Mos), cyclins, transcription factors (Maf2, GCN4, c-Jun, p53 and nuclear factor-κB), inhibitors of cyclin-dependent protein kinases (Sic1, Far1 and Rum1), and subunits of trimeric G-proteins [3,4]. Ubs are covalently ligated to proteins by the action of a multiple enzyme system consisting of E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes. 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 [5,6] and translocated into the central cavity of the 20 S proteasome, where they are degraded to small peptides in an ATP-dependent fashion [1-4,7].

Ubs are encoded by two distinct classes of genes, neither of which encodes a monomeric form of Ub [8,9]. One is a poly-Ub gene which encodes a linear polymer of Ubs that are 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 [10]. Thus generation of free Ub from linear Ub polymers and Ub-fusion proteins and recycling of Ub from poly-ubiquitinated protein conjugates by de-ubiquitinating enzymes (DUBs) should be essential for the Ub-mediated cellular processes.

DUBs can be divided into two groups: Ub C-terminal hydrolases (UCHs) and Ub-specific processing proteases (UBPs) [11]. As far as the UCH family is concerned, a neuron-specific UCH in the marine snail Aplysia has been reported to be essential for long-term facilitation [12]. Recently, Leroy and co-workers have identified, in a German family with Parkinson’s disease, a mutation in the UCH-L1 gene resulting in a change of Ile34 to Met, implicating the involvement of Ub pathway in Parkinson’s disease [13]. Siagoh et al. [14] have found that, in the gracile-axonal-dystrophy (gad) mouse showing ataxia, accumulation of amyloid β-protein and Ub-positive deposits occurs retrogradely along the sensory and motor nervous systems and that the gad mutation is caused by an in-frame deletion including exons 7 and 8 of UCH-L1. Johnston et al. [15] have determined the crystal structure of UCH-L3, which shows a central antiparallel β-sheet flanked on both sides by α-helices. This resembles the structure of the well-known papain-like cysteine proteases, with the greatest similarity to cathepsin B. As for the UBP family, many UBP s have been implicated in numerous cellular processes, such as eye development in the fly [16], transcriptional silencing [17], cytokine response [18], and growth regulation [18,19].

Abbreviations used: Ub, ubiquitin; UCH, ubiquitin C-terminal hydrolase; YUH1, yeast ubiquitin hydrolase 1; gal, galactosidase; UBP, ubiquitin-specific processing protease; USP, ubiquitin-specific protease; Unp, ubiquitously nuclear protein; DUB, de-ubiquitinating enzyme; UBP-PESTc, ubiquitin-aaH-MMISPPEPSSEEEHYC; CEP90, carboxyl extension protein 90; DHFR, dihydrofolate reductase; His-penta-Ub, poly-His-tagged penta-Ub; NLS, nuclear localization signal; Ni-NTA, Ni2+-nitrilotriacetate; DTT, dithiothreitol; Rb, retinoblastoma protein.

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In the mouse and human, cDNAs of ubiquitous nuclear protein (Unp) have been cloned. unp was originally identified during a survey of mouse genes near the Mv20 retroviral insertion site. Analysis of the Unp cDNAs has revealed their sequence homology with the UBP family, and their products have been shown to be functional DUBs [20–22]. In addition, the deduced amino acid sequences of the Unps contain putative nuclear localization signals (NLSs), based on their similarity to the NLS domain of the p53 protein [23], and the binding motifs for retinoblastoma protein (Rb) [24,25]. However, the subcellular localization of endogenous Unps has been controversial, since the mouse Unp has been reported to localize to the nucleus [26], while human Unp (Unph) has been shown to exist exclusively in the cytoplasm [27].

The mouse unp is related to the human oncogene tre-2 [27]. Expression of unp from a highly active promoter results in tumorigenic transformation of NIH3T3 cells, and induces lung carcinomas when the cells are injected into athymic mice [26]. Unph mRNA levels are consistently elevated in small-cell carcinomas when the cells are injected into athymic mice [26]. Unp (Unph) has been shown to exist exclusively in the cytoplasm [28], and its gene is localized to chromosome band 12q14-15 and 12q14-21 sequences has been amplified in 

\[ \text{Escherichia coli} \]

The mouse Unp (Unph) has been shown to exist exclusively in the cytoplasm [28], and its gene is localized to chromosome band 12q14-15 and 12q14-21 sequences has been amplified in cell lines, raising a possibility that Unps may be tumour suppressors. This contradictory observation for transforming potential and tumour-suppressing activity of Unps could be explained by an example of the tumour suppressor p53, which was originally identified as a proto-oncogene. Recently, a human Ub-specific protease gene, USP15, has been identified [28]. The USP15 protein has the sequence most closely related to Unp, and its gene is localized to chromosome band 12q14, a different location from that of unp (3p21.3). It has been reported that breaks in a broad region of chromosome 12, 12q13-q15, occur in lipomas [29]. In addition, amplification of 12q14-15 and 12q14-21 sequences has been found in the neuroblastoma cell line NGP [30] and both mucinous ovarian neoplasms and Brenner tumours respectively, which sometimes co-exist in the same patient [31].

Cloning of UB109 cDNA

The E. coli JM101 cells carrying both the rat cDNA plasmids and pACUb-R-β-gal were plated on to agar plates containing 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 40 μg/ml 5-bromo-4-chloroindol-3-yl β-d-galactoside (‘X-Gal’). They were then incubated at 37 °C for 14 h. Among 3 × 10⁵ transformants, three appeared as white colonies, while the others were stained as blue. All the control cells (i.e. transformed with pBS 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-R-β-gal or Ub-M-β-gal. Only one of the three colonies turned white when transformed with Ub-R-β-gal, but not with Ub-M-β-gal (i.e. became blue), confirming that the white colony contains the plasmid carrying the rat cDNA specifying the UBP activity against the Ub-β-gal proteins. The plasmid was isolated, sequenced, and is referred to as pBS-Ubp109. The UB109 cDNA was also cloned into pQE31 vector, and the resulting plasmid was referred to as pQE31-Ubp109.

In situ hybridization

In situ hybridization was performed essentially as described previously [40]. Radiolabelled riboprobes were synthesized by in vitro transcription using α-32P-UTP. Frozen embryo and adult tissues were cut into 12 μm-thick slices and mounted on to gelatin-coated slides. The sections were fixed in 4% (w/v) paraformaldehyde, treated with 0.25% (w/v) acetic anhydride in 0.1 M triethanolamine, pH 8, containing 0.9% (w/v) NaCl to reduce non-specific hybridization due to electrostatic forces, dehydrated and defatted in ethanol and chloroform, and finally air-dried. The hybridization solution (2 × 10⁵ c.p.m./ml) was applied directly to each mounted section for 16 h at 52 °C. The sections were washed in 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), treated with RNase A (20 μg/ml) for 30 min at 37 °C, and washed sequentially for 30 min in 1 × SSC and 0.5 × SSC at room temperature and 30 min in 0.1 × SSC at 60 °C. After drying, the slides were exposed for 7 days to β-max Hyperfilm (Amersham–Pharmacia) for autoradiography.

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Expression of UBP109 in E. coli

The XL-1/pQE31-Ubp109 cells were grown to mid-exponential phase in Luria broth at 37 °C. Expression of UBP109 was then induced for 2 h by treating with 1 mM isopropyl thio-β-D-galactoside. After the treatment, the cells were harvested and suspended in 50 mM phosphate buffer, pH 7.8, containing 0.3 M NaCl. The cell suspension was then disrupted in a French press at 96.6 MPa (14000 lb/in²) and centrifuged at 100000 g for 2 h. The resulting supernatants are referred to as ‘crude extracts’.

In vitro assays for UBP activity

The UBP activity was assayed by incubation of the reaction mixtures (0.1 ml) containing appropriate amounts of the enzyme fractions and 10 μg of 125I-labelled Ub-PESTc [(1–2) × 10⁶ c.p.m./μg] in 100 mM Tris/HCl (pH 8)/1 mM EDTA/1 mM dithiothreitol (DTT)/5% (v/v) glycerol [35]. After incubation for appropriate periods at 37 °C, the reaction was terminated by adding 50 μl of 40% (w/v) trichloroacetic acid and 50 μl of 1.2% (w/v) BSA. The samples were centrifuged, and the resulting supernatants were counted for their radioactivity using a γ-radiation counter. The enzyme activity is expressed as a percentage of 125I-labelled Ub-PESTc hydrolysed to acid-soluble products.

Preparation of poly-Ub–protein conjugates

To prepare poly-Ub–NH–protein conjugates, 1 μg of the 125I-labelled Ub [(1–2) × 10⁶ c.p.m./μg] was incubated with 720 μg of fraction II and an ATP-regenerating system [39]. The ATP-regenerating system consisted of 10 mM Tris/HCl (pH 7.8), 15 units/ml creatine phosphokinase, 6.5 mM phosphocreatine, 1.5 mM ATP, 1 mM DTT, 0.5 mM MgCl₂, and 1 mM KCl in a final volume of 0.3 ml. Incubations were performed for 2 h at 37 °C in the presence of 1 mM haemin to prevent proteolysis of the ubiquitinated protein conjugates by the 26 S proteasome. After incubation, the samples were heated for 10 min at 55 °C for inactivation of endogenous DUBs. The resulting samples were freed of excess 125I-labelled Ub by treatment with 0.2 N NaOH and 100 mM DTT, followed by gel filtration on a Sephadex G-75 column equilibrated with 25 mM Tris/HCl buffer, pH 7.8, containing 1 mM DTT and 1 mM EDTA.

Separation of cytosolic and nuclear fractions

L6 myoblasts were suspended in the hypo-osmotic buffer consisting of 10 mM Heps, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 mM KCl, and 2 mM PMSF, and placed on ice for 15 min. The samples were homogenized twice in a Dounce homogenizer and centrifuged for 5 min at 500 g and then for 30 min at 15000 g. The resulting supernatants are referred to as the ‘cytosolic fraction’. The pellets were resuspended in the same volume of the lysis buffer consisting of 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 2 mM PMSF. The samples were then centrifuged for 30 min at 15000 g. The resulting supernatants are referred to as the ‘nuclear fraction’.

Immunoblot analysis

To prepare polyclonal antiserum against UBP109, a multiple antigenic peptide, CSEMETDEPDDESQDQE, derived from the internal sequence (625–641 residues) of the protein was synthesized (Nippon Roche Research Center, Kamacura City, Japan). This peptide, conjugated to keyhole-limpet haemocyanin, was subcutaneously injected into albino rabbits. From the antiserum, the anti-UBP109 IgGs were affinity-purified using the peptide linked to CNBr-activated Sepharose-4B (Sigma). For immunoblot analysis, SDS/PAGE was carried out using the cytosolic and nuclear fractions obtained from L6 myoblasts. Proteins in the gels were transferred on to nitrocellulose membranes and incubated with the purified anti-UBP109 IgG [41]. The membranes were then incubated with the rabbit anti-IgG conjugated with horseradish peroxidase (HRP), and immuno-reactive bands were detected using 4-chloro-1-naphthol or an enhanced chemiluminescence (ECL*) kit (Amersham–Pharmacia).

Determination of subcellular localization of UBP109

In order to generate mutations in three putative NLSs of UBP109, mutagenesis was performed by PCR to replace 531RKKP, 671HKKR and 728LKRR by RIDP, HEDP and IGER respectively, using oligonucleotide primers. The UBP109 cDNAs carrying each mutation were ligated into pcDNA3.1 and tagged with myc to their 5′-ends of the open reading frame. The resulting plasmids carrying the mutations in the first, second and third sites are referred to as pcDNAmyc109-1, -2 and -3 respectively, and the plasmid containing the wild-type UBP109 cDNA is referred to as pcDNAmyc109.

NIH3T3 cells and L6 myoblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml of penicillin and 1 μg/ml of streptomycin. The culture medium also contained 10% (v/v) calf serum for NIH3T3 cells or 10% (v/v) fetal bovine serum for L6 myoblasts. Cells were then plated at 4 × 10⁴ cells/ml density on gelatin-coated glass coverslips. After culturing for 24 h, the cells were transfected with the recombinant pcDNA3.1 plasmids using LipofectAMINE PLUS (Gibco BRL) by following the procedure recommended by the manufacturer. The transfected cells were cultured for 3 h, re-fed with the fresh culture medium supplemented with 10% serum, and further cultured for 24–48 h.

For cell staining, the cultured cells on coverslips were fixed for 10 min in 3.7% (w/v) paraformaldehyde buffered with PBS and permeabilized with PBS containing 0.5% (v/v) Triton X-100 for 5 min. The coverslips were then blocked with PBS containing 10% (v/v) goat serum and 0.1% (w/v) gelatin, and incubated for 1 h with anti-myc monoclonal antibody in PBS containing 1% BSA. They were washed three times with PBS containing 0.1% Triton X-100. Stained cells were mounted on slide glasses with Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.) and observed with a laser scanning confocal microscope (Carl Zeiss LSM 510) [42]. Samples were excited by a 488 nm argon laser, and the images were filtered by a long-pass 505 nm filter.

RESULTS

Cloning of ubp109

Using an E. coli-based in vivo screening methods, we isolated a cDNA expressing de-ubiquitinating activity from rat skeletal-muscle cDNA library. The open reading frame specifying the de-ubiquitinating activity was identified by subcloning and nucleotide sequencing of the cDNA and named ubp109, since it encodes a 109 kDa protein (GenBank accession number AF106657). This cDNA has a stop codon (TAG at –12) within the short 5′ untranslated region and Kozak consensus sequence (AAG-ATGG) [43]. The position of the start (ATG) codon of
Figure 1  Nucleotide sequence of the UBP109 cDNA and deduced amino acid sequence of the UBP109 protein

The nucleotides are numbered on the right, beginning at A of the presumed start codon. The amino acid residues are numbered on the right. The asterisk indicates the TAG stop codon. The regions with the bold characters are the conserved Cys, Asp, and His domains, the boxed regions are the putative NLS motifs, and the underlined regions are the Rb-binding motifs.

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ubp109 was inferred to yield the 2856 bp open reading frame, which encodes a protein consisting of 952 residues (109 255 Da) (Figure 1). From the deduced amino acid sequences, the pI was estimated to be 4.98.

Sequence alignment demonstrated that UBP109 had the Cys and His domains, which is typical for DUBs, and showed 98.2 % amino acid sequence identity with USP15, which is a recently reported human UBP, and about 60 % identity with Unps of human and mouse, which also are UBPs. Thus UBP109 is a rat homologue of human USP15. The deduced amino acid sequence contained three putative NLSs, based on their similarity to the NLS domain of p53 protein [23]. In addition, three motifs common to proteins that physically associate with Rb protein were also found (Figure 1).

Expression of UBP109 mRNA

To investigate the expression of UBP109 mRNA, Northern-blot analysis was performed using the 32P-labelled 5'-region of Ubp109 (367 bp fragment) as a probe. A single transcript of about 3.7 kb was detected in various rat tissues. The size of the UBP109 cDNA, consisting of 2856 bp and poly(A) tail, corresponded well with the estimated size of the transcript (Figure 2A). Interestingly, expression of the UBP109 mRNA was the highest in testis and spleen, although it was also detected in the other tissues tested.

The UBP109 protein is the sequence most closely related to Unp, a candidate for tumour suppressor, sharing about 60 % sequence identity. The gene for USP15, a human homologue of rat UBP109, is localized to chromosome band 12q14, a region where breakage or amplification in various carcinomas has been reported. In an attempt to determine the relationship of UBP109 with cancer, we examined the expression of UBP109 mRNA in various carcinoma cell lines (Figure 2B). The UBP109 mRNA was expressed to various extents among the samples, to high levels especially in lymphoma cells (U937), but there was little or no expression in cervical carcinoma (HeLa), hepatoma (HepG2) and neuroblastoma (SH-SY5Y) cells. Interestingly, an extra faint band was detected only in non-transformed cells, such as L6 myoblasts and FLF human primary fibroblast cells (indicated by an asterisk). However, it remains unknown whether the extra
band represents an isoform of UBP109 or a differently spliced product.

Upon in situ hybridization we also localized the UBP109 transcript on the parasagittal whole-body sections of 12–20-day-old rat embryos (referred to as E12–E20), as well as on adult tissue sections (Figure 3). Expression of the UBP mRNA in the liver was dramatically increased during the E14–E16 stage (Figures 3B and 3C) and gradually reduced thereafter. In the spinal cord, the mRNA level reached a maximum at E16 (Figure 3C) and rapidly declined during the E18–E20 stage (Figures 3D and 3E). The signal in the brain was also detected from E16, but declined more slowly than that in the spinal cord at the later stage. On the other hand, the UBP109 mRNA began to appear in the intestine at E20 (Figure 3E). Little or no UBP109 mRNA was detected when the same experiments were performed using a sense probe (Figures 3F–3J). Interestingly, expression of the UBP109 mRNA was localized almost exclusively to the outer layer of the intestine in the adult tissue (Figure 3K) as well as in the embryo (Figure 3E). In accord with the Northern-blot data, both of the adult spleen and testis tissues strongly expressed the UBP109 transcript (Figures 3K and 3L). On the other hand, little or no signal was detected in the other adult tissues, unlike the results of Northern-blot analysis, which used concentrated RNA preparations. These results suggest that expression of the UBP109 mRNA is regulated during embryonic development and is tissue-specific in adults.

**Table 1 Summary of the purification of UBP109**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1000</td>
<td>37,024</td>
<td>37</td>
<td>100</td>
</tr>
<tr>
<td>NTA–agarose</td>
<td>15</td>
<td>12,308</td>
<td>1026</td>
<td>33</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
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<td>10,837</td>
<td>9031</td>
<td>29</td>
</tr>
<tr>
<td>CM-Sepharose</td>
<td>0.5</td>
<td>6875</td>
<td>13,750</td>
<td>18</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>0.2</td>
<td>3080</td>
<td>15,385</td>
<td>6.3</td>
</tr>
</tbody>
</table>

To purify the UBP109 protein, crude extract (1 g) was prepared from 12 g of *E. coli* cells that had been transformed with pQE31-Ubp109. During purification, the enzyme activity was monitored by determining its ability to hydrolyse 125I-labelled Ub-PESTc. The extracts were applied to an NTA–agarose column (1 cm × 5 cm) equilibrated with 30 mM phosphate (pH 7.8)/0.3 M NaCl. The column was washed with the phosphate buffer containing 35 mM imidazole, adjusted to pH 6.8. Proteins bound to the column were eluted with a linear gradient of 35–200 mM imidazole. Fractions with high activity were pooled and dialysed against buffer A, and the column was washed with the same buffer. The bound proteins were eluted with a linear gradient of 0–300 mM NaCl. Fractions with high activity were pooled and dialysed against 20 mM phosphate buffer, pH 6.5, containing 5 mM 2-mercaptoethanol, 1 mM EDTA and 10% glycerol. The dialysed sample was applied to a CM-Sepharose column (1 cm × 5 cm) equilibrated with buffer A, and the column was washed with the same buffer. The bound proteins were eluted with a linear gradient of 0–300 mM NaCl. Fractions with high activity were pooled and dialysed against 20 mM phosphate buffer, pH 6.5, containing 5 mM 2-mercaptoethanol, 1 mM EDTA and 10% glycerol. The column was washed with phosphate buffer, pH 7.8, containing 5 mM 2-mercaptoethanol, 1 mM EDTA and 10% glycerol. The dialysed sample was applied to a CM-Sepharose column (1 cm × 5 cm) equilibrated with the phosphate buffer. After washing the column, proteins bound to the column were eluted with a linear gradient of 50–350 mM phosphate. Fractions with high activity were pooled and dialysed against buffer A. The dialysed sample was applied to a Q-Sepharose column (1 cm × 5 cm) equilibrated with buffer A, and the bound proteins were eluted with a linear gradient of 0–300 mM NaCl. Fractions under the symmetric peak of the Ub-PESTc-degrading activity (Figure 4A) were pooled, dialysed against buffer A, concentrated by ultrafiltration, and kept frozen at −70 °C until use. A summary of the purification protocol is shown in Table 1. The size of the enzyme was estimated to be about 120 kDa upon chromatography on a Sephacryl S-300 column (1 cm × 30 cm) equilibrated with buffer A containing 0.1 M NaCl (results not shown). It also ran as a single protein of 118 kDa upon analysis by SDS/PAGE (Figure 4B). Since the enzyme was expressed as a poly-His-tagged fusion protein, it ran larger than 109 kDa under both non-denaturing and denaturing conditions. These results indicate that UBP109 consists of a single polypeptide.
Biochemical properties

To determine the time-dependent hydrolysis of 125I-labelled Ub-PESTc by the purified UBP109, assays were performed by incubation of the enzyme at 37 °C for various periods. Poly-L-Lys has been shown to markedly stimulate the Ub-PESTc-hydrolysing activity of YUH1 as well as of most UCHs identified in chick skeletal muscle [35]. Therefore the enzyme assay was also performed in the presence of 10 μg of poly-L-Lys. As shown in Figure 5(A), the polycationic agent activated the enzyme activity nearly 100-fold. In addition, the rate of Ub-PESTc hydrolysis increased linearly with time at least for 1 h under the assay conditions. This extent of activation was much higher than that observed with any other DUBs, including YUH1 or chick UCH6 [35]. Using a double-reciprocal plot of the data, the K_m for Ub-PESTc was estimated to be 1.67 and 104 μM in the presence and absence of poly-L-Lys respectively (Figure 5B). On the other hand, little or no change was observed for the V_max value upon treatment with poly-L-Lys. These results indicate that the activation of the enzyme activity by poly-L-Lys is due to a marked increase in the affinity of the Ub-PESTc substrate to UBP109.

We also examined the effects of various protease inhibitors. N-ethylmaleimide (1 mM), a thiol-group-blocking agent, almost completely blocked the activity of UBP109 against Ub-PESTc. Ub-aldehyde (1 μM), which is a specific inhibitor of DUBs, also strongly inhibited the enzyme activity. On the other hand, little or no effect was observed upon treatment with o-phenanthroline, a metal chelating agent, or PMSF, which is a serine-protease inhibitor (results not shown).

Hydrolysis of Ub precursors and poly-Ub–protein conjugates

In order to determine the substrate specificity of UBP109, the purified enzyme was incubated with various Ub-α-NH-protein extensions, such as Ub-DHFR, Ub-CEP80 and His-penta-Ub. After incubation, the samples were subjected to SDS/PAGE using 4, 10, and 16% discontinuous slab gels as described previously [35], followed by staining with Coomassie Blue R-250. As a control, hydrolysis of Ub-PESTc was also determined under the same conditions. UBP109 was capable of generating free Ub from all of the substrates tested (Figure 6A). To determine whether UBP109 can release free Ub from Ub-M-β-gal under in vitro conditions, the purified enzyme was incubated with the extracts of E. coli MC1000 cells that had been transformed with pACUb-M-β-gal. The samples were then subjected to immunoblot analysis using an anti-β-gal antibody. Figure 6(B) shows that UBP109 can rapidly hydrolyse Ub-M-β-gal. These results indicate that the purified UBP109 is capable of cleaving the C-terminus of the Ub moiety in natural and engineered fusions irrespective of their sizes.

We then examined whether UBP109 could also release free Ub from poly-Ub-α-NH-protein conjugates. The substrates were prepared using 125I-labelled Ub and fraction II of rabbit reticulocytes and incubated with the purified UBP109 followed by SDS/PAGE. As shown in Figure 6(C), the amount of high-molecular-mass poly-Ub–protein conjugates was significantly decreased with a concomitant increase in the amount of Ub band upon incubation in the presence of the enzyme compared with that in its absence (Figure 6, lanes b and c respectively). On the other hand, treatment with N-ethylmaleimide (1 mM) or Ub-aldehyde (1 μM) almost completely prevented the generation of free Ub (Figure 6, lanes d and e respectively). These results indicate that UBP109 is capable of releasing free Ub from branched poly-Ub chains conjugated to proteins.

Subcellular localization of UBP109

UBP109 has three putative NLS motifs (see Figure 1). To determine the subcellular localization of UBP109, we first separated the lysates of cultured L6 myoblasts into cytosolic and nuclear fractions. The same aliquots of the fractions were then subjected to immunoblot analysis using the purified anti-UBP109 IgGs. As shown in Figure 7(A), the intensity of the UBP109 protein band in the cytosolic fraction was about the same as that in the nuclear fraction. Tubulin-β protein is shown as a cytosolic marker protein. These results strongly suggest that UBP109 localizes to both the cytoplasm and the nucleus.
In order to clarify further the localization of UBP109, NIH3T3 cells were transfected with pcDNAmyc109 expressing myc-tagged UBP109. The cells were then subjected to immunostaining with an anti-myc antibody, followed by confocal microscopy. Figure 7(B) shows that the cells are stained in the nucleus as well as in the cytoplasm, confirming that UBP109 is localized to both of the subcellular fractions. Similar data were obtained when the same experiment was performed using L6 myoblasts (results not shown). To find out which of the three putative NLS motifs is responsible for nuclear translocation of UBP109, we generated mutations in each of the motifs. The mutated cDNAs in pcDNA3.1 were transfected into NIH3T3 cells, followed by confocal microscopy. Transfection of pcDNAmyc109-1 and -2, in which RKKP and HKKR were replaced by RIDP and HEDP respectively, did not show any effect on the localization of UBP109 (Figures 7C and 7D respectively). Strikingly, the enzyme was exclusively localized to the cytoplasm of the cells transfected with pcDNAmyc109-3, in which LKKR was substituted with IDER (Figure 7E). These results indicate that the third NLS motif (LKKR) is responsible for nuclear localization of UBP109.

DISCUSSION

In the present study we have cloned a cDNA that encodes the 109 kDa UBP protein, UBP109, from rat skeletal-muscle cDNA library. We have also purified the protein to apparent homogeneity upon expression in E. coli and demonstrated that the purified enzyme is capable of releasing free Ub, not only from linear Ub–protein conjugates, such as Ub-CEP80, Ub-DHFR, His-penta-Ub, and Ub-β-gal, but also from branched poly-Ub–protein conjugates. Thus it appears likely that UBP109 plays an important role in generation of free Ub molecules from its precursors and in recycling and/or ‘trimming’ of Ub from poly-Ub–protein conjugates. Of interest is the finding that poly-lys-Lys dramatically stimulates the activity of UBP109 on Ub-PESTc. Its stimulatory effect appears to be due to an increase in the affinity of the substrate for the enzyme, perhaps through neutralizing the negative charge in the ‘PEST’ sequence of Ub-PESTc fusion. However, the mechanism for the activation of the UBP109 activity by poly-lys-Lys remains unclear, because other polycationic agents, such as histone, spermine and putrescine, show little or no effect on the enzyme activity, similar to the chick UCH6 that is activated by poly-lys-Lys but not the others [35].

Recently, Baker and co-workers [28] have cloned an UBP cDNA using KIAA0529 human cDNA and designated its product as USP15. They also showed that USP15 has about 60% sequence identity with human and mouse Unps. Amino-acid-sequence analysis has revealed that the rat UBP109 shows as much as 98.2% sequence identity with human USP15. Thus UBP109 is a homologue of human USP15, and these two enzymes are closely related to the human and mouse Unps but are not their homologues. Interestingly, all of these UBP enzymes contain three putative NLS motifs in addition to multiple Rb-binding sequences and the conserved Cys/His domains that are a characteristic of DUBs. Their short, putative NLS sequence is a typical XKKX, where X is any residue, as has been found in the RC3 and RC9 subunits of rat proteasome [44–46] and subunit YC7α of yeast proteasome [47], and appears sufficient for their nuclear translocation [48]. The presence of this motif is consistent with our finding of UBP109 in the nuclear fractions of L6 myoblasts. However, contrary to the report that human Unp exclusively localizes to the cytosolic fraction of HeLa cells and that mouse Unp exists predominantly in the nucleus of NIH3T3 cells [22,26], UBP109 was found in both the nucleus and the cytoplasm of NIH3T3 cells, as well as of L6 myoblasts. Moreover, we found that, among the three putative NLSs only the one located nearest to the C-terminus was responsible for nuclear localization.
Tissue-specific expression of rat protease UBP109

Figure 7 Subcellular localization of UBP109 in cells

L6 myoblasts cultured for 72 h (A) were harvested and fractionated into cytosolic and nuclear fractions as described in the Experimental section. The same volume of each fraction was subjected to SDS/PAGE as in Figure 4, followed by immunoblot analysis using the purified anti-UBP109 IgG. Lane c and lane n indicate the cytosolic and nuclear fractions, respectively. β-Tubulin was also used as a cytosolic marker protein. NIH3T3 cells were transfected with pcDNAmyc109 (B), pcDNAmyc109-1 (C), pcDNAmyc109-2 (D) or pcDNAmyc109-3 (E). After transfection, the cells were stained and observed under a confocal laser scanning microscope as described in the Experimental section.

translocation of UBP109. Perhaps the different localization of the Unp and UBP109 family reflects the specific role of each enzyme in different tissues with different substrates.

Of particular interest is the finding that the expression of UBP109 transcript is developmentally regulated upon analysis by in situ hybridization. In the liver and the spinal cord, the UBP109 mRNA was strongly expressed during E12–E14 and disappeared at the later stage of embryonic development. In the brain, the signal began to appear at E16 and became more intense at the later stages. However, the UBP109 mRNA could be detected in none of the adult tissues. On the other hand, in the intestine, the UBP109 mRNA was detected from E20 and more intensely at adult stage. Thus it appears likely that the UBP109 enzyme plays an important role during embryonic development, although its specific function in each organ is unknown.

Moreover, the extremely high expression of UBP109 mRNA in specific adult organs such as the spleen and the testis implies specific functions of the enzyme in those tissues. As for the testis, UBP109 may be involved in cell proliferation or spermiogenesis. Recent evidence suggests that the Ub/proteasome system is involved in the loss of most of the cytoplasm occurring during spermiogenesis [49,50]. UBP109 may activate the proteasome-mediated degradation of the cytoplasmic proteins by generation of free Ub from its precursors and/or recycling or ‘trimming’ of Ub from poly-Ub–protein conjugates. The abundance of UBP109 mRNA in both the fetal liver and the adult spleen could also suggest its additional role in haematopoiesis. There have been several reports of DUBs involved in haematopoiesis, such as murine DUB-1, which shows significant similarity with Unps and is proposed to be a product of hematopoietic-specific immediate early gene induced by interleukin-3 and interleukin-5 [18,51]. In addition, the recently reported UBP43 [52], which is highly expressed in fetal liver, has also been suggested to play an important role in hematopoietic cell differentiation. UBP109 may also involve in hematopoiesis occurring initially in the fetal liver and then in adult spleen. Since during haematopoiesis a common stem cell becomes committed to differentiate along particular lineage by specific cytokines, and since specific cytokines induce specific genes, which include some UBP genes, it seems possible that different cytokines induce different UBPs with distinct substrate specificity and that the induced UBPs cleave different substrates involved in the formation of various types of the blood cells.

The gene for USP15, a human homologue of rat UBP109, has been shown to localize to chromosome band 12q14, a different location from that for human Unps (3p21.3). In many carcinomas, disturbance of chromosome 12 is frequently found. Breaks occur in a broad region of chromosome 12, 12q13-q15, in lipomas [29]. Amplification of 12q14-15 and 12q14-21 sequences has also been found in the neuroblastoma cell line NGP [30] and in both mucinous ovarian neoplasms and Brenner tumours respectively, which sometimes co-exist in the same patient [31]. In addition, UBP109 was found to show approx. 60% sequence identity with human Unph, which is proposed to be a proto-oncogene product related to tre-2, leading us to consider a possible involvement of UBP109 in tumorigenesis. Although we have no evidence connecting UBP109 to cancer, the expression of UBP109 mRNA was found to vary markedly among different carcinomas, compared with non-transformed cells, including human primary fibroblasts and rat L6 myoblasts. For example, the level of UBP109 mRNA is strongly expressed in human
premyeloma (U937), whereas little or none is detected in cervical carcinoma (HeLa), hepatoma (HepG2) and lung carcinomas (H1299). In addition, it is noteworthy that a smaller transcript, which reacts with the UBPI09 cDNA probe, is present in the non-transformed cells but not in any of the tumour cell lines tested (see Figure 2B). Thus it is tempting to speculate that UBPI09 may be involved in the regulation of cell growth, although we do not have any evidence that there exists any aberration of the chromosome 12q14 region in the carcinoma cell samples we used.

The presence of Rb-binding motifs in UBPI09 also suggests a possible role of the enzyme in cell-growth regulation. Recent evidence has shown that Rb is a target for ubiquitination [53]. UBPs containing Rb-binding motifs, including UBPI09, may act as a proto-oncogene product through participation in the Rb degradation and in turn overcoming the inhibitory effect of Rb on the cell cycle. On the other hand, there is a report showing that the level of Unp in lung-carcinoma cell lines is rather decreased, compared with normal cells [22]. Moreover, Unp has been reported to interact with Rb in mouse cells as well as under in vitro conditions [28], suggesting a possibility that Unps are involved in stabilization of Rb rather than its degradation and hence in cell-growth regulation. Thus it is again tempting to speculate that UBPI09 may also participate in regulation of cell growth through an Rb-mediated pathway, although the binding of UBPI09 with Rb has not yet been corroborated.

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