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Isolation and Characterization of Cytosolic and Membrane-Bound Deubiquitinylating Enzymes from Bovine Brain[1]

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The deubiquitinylating enzymes (DUBs), that release free ubiquitin (Ub) from its precursors or ubiquitinylated proteins, are known to comprise of a large protein family in eukaryotes, but those in mammalian tissues remain largely unknown. Here we report the existence of unexpectedly large species of DUBs in both soluble and membrane-bound fractions of bovine brain, based on their ability to cleave ^{125}I-labeled Ub-fused αNH-MHISPPEPESEEEEEHYC (designated as Ub-PESTc). Two cytosolic enzymes, tentatively called sDUB-1 and sDUB-2, with molecular masses of about 30 kDa were purified to near homogeneity by Ub-Sepharose affinity chromatography. sDUB-1 and sDUB-2 corresponded to UCH-L3 and UCH-L1/PGP 9.5, respectively. Intriguingly, the particulate fraction of the brain homogenate was found to also contain strong activities against ^{125}I-Ub-PESTc, which can be solubilized by treatment with 5% *n*-heptyl-β-D-thioglucoside and 1% Nonidet P-40, but not by washing with 1 M NaCl. From the solubilized material, two new 30-kDa, membranous DUBs (called mDUB-1 and mDUB-2) were purified to apparent homogeneity by Ub-Sepharose chromatography. Two other Ub-aldehyde sensitive DUBs, designated as mDUB-3 and mDUB-4, were also partially purified by conventional chromatographic operations. These mDUBs differed from each other in substrate specificity and exhibited different characteristics from the sDUBs, revealing that they are a new type of membrane-bound DUB. These results indicate the presence of divergent DUBs in mammalian brain, which may contribute to regulation of numerous pivotal cellular functions mediated by the covalent modification of Ub.

Key words: bovine brain, deubiquitinylating enzyme, ubiquitin, ubiquitin hydrolase, ubiquitin specific protease.

Ubiquitin (Ub) is a highly conserved 8.6-kDa polypeptide. It is ligated to a variety of target proteins *via* an isopeptide linkage through the action of a multi-enzyme system consisting of E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes (*1, 2*). This covalent modification of ubiquitinylation has been indicated to play critical roles in various biologically important processes, such as DNA repair, proteolysis, endocytosis and so on (*1, 3*). The best-characterized modifying reaction is the formation of a poly-Ub chain functioning as a signal for the proteolytic attack by the proteasome (*2, 4*).

It is notable that Ub is not degraded during proteasome-mediated intracellular protein breakdown but reutilized in cells (*5*). In yeast, Ubp4 (also known as Doa4) is responsible for the removal of peptides from the poly-ubiquitinylated intermediates generated through the action of the proteasome on poly-ubiquitinylated proteins (*6*). On the other hand, disassembly of free poly-Ub chains is catalyzed by Ubp14 in yeast and the homologous isopeptidase T in mammalian cells (*7*). In addition, an isopeptidase associated with the 26S proteasome has been proposed to act as a "proofreading" or "editing" enzyme controlling the ubiquitinylated state of proteins or disassembly of degradation

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Abbreviations: Ub, ubiquitin; Ubl, ubiquitin-like protein; Ub-CHO, ubiquitin aldehyde; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SUMO1, small ubiquitin-related modifier 1; NEDD8, neural precursor cell-expressed developmentally down-regulated gene 8; DUB, deubiquitinylating enzyme; UCH, ubiquitin carboxyl-terminal hydrolase: UBP, ubiquitin-specific protease; NP-40, Nonidet P-40; DTT, dithiothreitol; PESTc, αNH-MHISPPEPESEEEEEHYC; NEM, *N*-ethylmaleimide; PCR, polymerase chain reaction.

intermediates (*8*). Thus, these deubiquitinylating enzymes (DUBs) are likely to play an essential role in facilitated Ub-dependent proteolysis mediated by the proteasome.

Ub is encoded by two types of unique genes. One is the poly-Ub gene encoding a tandemly repeated Ub structure, which is a so-called "heat-shock gene" responsible for the rapid and efficient production of large amounts of Ub molecules in cells in response to various environmental stresses (*9*). The other Ub gene produces a Ub fused with certain ribosomal proteins, of which the biological significance remains unknown, although it is suspected to be responsible for determining the balance between the degradation and synthesis of cellular proteins (*2*). These gene structures indicate that cells contain an enzymatic system(s) that generates free Ub molecules efficiently from their precursors. Indeed, poly-Ub proteins and/or Ub-fused proteins are hardly detected in cells, indicating that the enzymes responsible for the maturation of Ub precursors are very active in cells (*5, 10*). However, the identity of the DUBs specific for processing of Ub precursors remains unknown at present.

DUBs are known to comprise of a large protein family in eukaryotes. For example, 17 genes for DUBs are present in the budding yeast genome (*2*). Moreover, more than 60 full-length DUB sequences have been identified in various eukaryotic cells so far (*10*). However, it remains to be determined how many DUBs are present in mammalian cells. The reason why a comprehensive search for cellular DUBs has not yet been conducted seems to be due to the lack of an efficient method(s) for assaying the activity of DUBs *in vitro*. We recently reported that ^{125}I-labeled Ub-fused αNH-MHISPPEPESEEEEEHYC (designated as Ub-PESTc) acts as a substrate for the sensitive and quantitative assaying of various DUBs, and found a set of novel DUBs in chick skeletal muscle (*11-14*), although little is known about their biological roles.

Intriguingly, many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, ALS (amyotrophic lateral sclerosis) and Huntington's disease, have been documented involving abnormal accumulation of Ub or ubiquitinylated proteins in the human brain, implying the dysfunction of the Ub metabolic pathway in regulating the dynamic state of the Ub pool in the tissue (*15, 16*). Therefore, it should be of importance to study the DUBs in the brain in order to ascertain whether or not the DUB genes have any relation with certain genetic disorder(s). With this background, the present study was performed to characterize DUBs in the bovine brain using ^{125}I-labeled Ub-PESTc as a substrate. Here we report that a large number of DUBs are present in the cytosol of the bovine brain. Surprisingly, we also found several novel DUBs that appear tightly associated with the membranes of the brain. The properties and possible biological significance of some of these DUBs are discussed.

MATERIALS AND METHODS

Materials—The compounds used were as follows: Ub (Sigma), hydroxylapatite HTP (Bio-Rad), Q-Sepharose FF, and Hitrap-Heparin-Sepharose CL-6B (Pharmacia Biotech), *n*-heptyl-β-D-thioglucoside (Dojindo), NP-40 (Nakalai Tesqus), anti-human PGP 9.5 antibody (Ultraclone, RA95101), and anti-p53 antibody (Calbiochem). Ub-aldehyde (Ub-CHO) and Ub-Sepharose were prepared as described previously (*11, 17, 18*).

Preparation of Recombinant Ub and Ubl (Ub-Like Protein) Fused with the PESTc Sequence—The recombinant Ub-PESTc was prepared as reported previously (*11*). The DNA fragments encoding Ubl (SUMO1 and NEDD8)-gsPESTc were synthesized by PCR and ligated into the expression vector, pGEX-2T (Pharmacia Biotech). Note that GS-PESTc (abbreviated as gsPESTc) was used instead of PESTc for PCR construction. For preparation of these Ubl-substrates, we used Ubl precursor forms; *i.e.*, (mature SUMO1-GG)HSTV fused with gsPESTc and (mature NEDD8-GG)LRQ fused with gsPESTc were constructed, and abbreviated as SUMO1-gsPESTc and NEDD8-gsPESTc, respectively. The recombinant GST-fused SUMO1-gsPESTc or NEDD8-gsPESTc proteins expressed in *Escherichia coli* were purified by GSH-Sepharose affinity chromatography and then digested with thrombin to remove the N-terminal GST moiety. The cDNAs encoding Ub-gsPESTc and Ub(G76A)-gsPESTc (Gly at position 76 of the C-terminal end of Ub was replaced by Ala) were synthesized by PCR and ligated into the pET3a vector. The recombinant Ub-gsPESTc and Ub(G76A)-gsPESTc proteins were purified by heat tretament (80°C for 10 min), $(NH_4)_2SO_4$ (65-90%) fractionation, and Q-Sepharose chromatography.

Radioiodination of Ub-PESTc, NEDD8-gsPESTc, and SUMO1-gsPESTc—The purified recombinant proteins were radiolabeled with Na^{125}I using IODO-BEADS (Pierce) according to the manufacturer's recommendation. The specific activity of iodinated Ub-PESTc and Ubl-PESTc was about 5×10^5 cpm/mg protein.

Assaying of Deubiquitinylating Activity—^{125}I-Ub-PESTc, ^{125}I-NEDD8-gsPESTc, or ^{125}I-SUMO1-gsPESTc (0.5-1 μg) was incubated at 37°C for 1 h in a total volume of 0.1 ml of a reaction mixture comprising 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol (DTT), 1 mM EDTA, 5% (v/v) glycerol, and a proper amount of DUB in the presence or absence of poly-L-Lys (10 μg/ml). For assaying quantitatively the degradation of ^{125}I-labeled fusion proteins, the radioactivity in acid-soluble products was determined as described previously (*11*). To validate the generation of Ub or Ubl (*i.e.*, SUMO1 or NEDD8) from the fusion protein(s), the reaction products were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by staining with Coomassie Blue R-250 or autoradiography. The intensity of the resulting bands was determined with a Fuji BAS-2500 Bio-imaging analyzer. When the Ub-carboxyl extension protein of 80 amino acids (CEP80), Ub-dihydrofolate reductase (DHFR), and poly-His-tagged di-Ub (His-di-Ub) were used as substrates, the reaction products were subjected to SDS-PAGE followed by staining with Coomassie Blue R-250 (*11*).

Chromatographic Operations—All chromatographic procedures were performed at 4°C, unless otherwise specified. Buffer-A comprised 20 mM Tris-HCl (pH 8.5), 1 mM DTT, and 1 mM EDTA, and Buffer-B comprised 10 mM K-phosphate (pH 6.8) and 1 mM DTT.

Other Biochemical Analysis—SDS-PAGE was carried out on 12.5 or 15-25% gradient slab gels (*19*). Proteins in the gels were visualized by staining with Coomassie Blue R-250. Immunoblot analysis was performed as described by Towbin *et al.* (*20*). Protein concentrations were mea-

sured by the method of Bradford (*21*) with bovine serum albumin as a standard. For determination of proteins in the presence of non-ionic detergents, a BCA (bicinchoninic acid) Protein Assay Kit (Pierce) was used. To determine the internal amino acid sequences of purified proteins, their fragments obtained on digestion with lysylendopeptidase were purified by reverse-phase high-performance liquid chromatography (HPLC). The purified peptides were then subjected to automated Edman degradation.

RESULTS

Separation of Soluble Enzymes Degrading Ub-PESTc from Bovine Brain—Whole bovine brains (approximately 400 g) were homogenized in 3 volumes of 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM DTT in a Potter-Elvehjem homogenizer. After centrifugation of the homogenates for 10 min at $1,000\times g$, the supernatants were removed from the precipitates (see below). The resulting supernatants were again centrifuged for 1 h at $10,000\times g$ and then for 1 h at $100,000\times g$. After these centrifugations, approximately 8.1 g of cytosolic proteins was obtained. These extracts rapidly hydrolyzed ^{125}I-Ub-PESTc into acid-soluble products, and this activity was almost completely inhibited upon treatment with 1 μM Ub-CHO, a typical inhibitor of DUB (*20*), or 1 mM *N*-ethylmaleimide (NEM), a sulfhydryl-blocking reagent (data not shown). These results indicate that the cleavage site in Ub-PESTc lies in the peptide bond between Ub and PESTc, and that a free sulfhydryl residue is required for the activity, similar to in the cases of the other known DUBs.

A part of the cytosolic proteins (1.6 g protein) was mixed with Q-Sepharose resin (70 ml) that had been equilibrated with Buffer-A. The mixtures were packed into a column (3.5×25 cm) and washed with more than 5 bed volumes of Buffer-A. Proteins adsorbed to the resin were then eluted with 560 ml of a linear gradient of 0-0.8 M NaCl in the same buffer. Figure 1A shows that the ^{125}I-Ub-PESTc-degrading activities were eluted as two peaks, named peak-1 and peak-2 in order of their elution.

To ascertain whether or not the conversion of the radioactivity of ^{125}I-Ub-PESTc into acid-soluble products indeed corresponds to the deubiquitinylating activity, we directly analyzed the production of the Ub moiety by the electrophoretic method described under "MATERIALS AND METHODS." Figure 1A (lower panel) shows that the profile of the disappearance of ^{125}I-Ub-PESTc is in accord with that of the increase in acid-soluble radioactivity (upper panel). The production of ^{125}I-Ub also occurred in parallel with the loss of ^{125}I-Ub-PESTc. However, the intensity of the band corresponding to Ub was much weaker than that for Ub-PESTc, because under the labeling conditions used the PESTc portion of the fusion protein was preferentially radioiodinated (*11*).

We then examined whether or not the column fractions are also capable of generating SUMO1 and NEDD8 upon incubation with ^{125}I-SUMO1-gsPESTc and ^{125}I-NEDD8-gsPESTc. As shown in Fig. 1A (lower panel), the elution pattern of the activity against ^{125}I-NEDD8-gsPESTc exhibited at least three peaks. The strong activity observed around fraction Nos. 55-64 resembled that against ^{125}I-Ub-PESTc, suggesting that the same enzymes may be responsible for the hydrolysis of both Ub-PESTc and NEDD8-gsPESTc. This possibility is supported by the fact that among various Ubls, NEDD8 shows the highest similarity to Ub in amino acid sequence, exhibiting about 60% identity. However, two other activities against ^{125}I-NEDD8-gsPESTc were observed around fraction Nos. 31-34 and 40-43, which showed considerably low activity against ^{125}I-Ub-PESTc, indicating that the fractions may contain specific enzymes for the cleavage of ^{125}I-NEDD8-gsPESTc. It is of note that considerable activity against both ^{125}I-Ub-PESTc and ^{125}I-NEDD8-gsPESTc was also

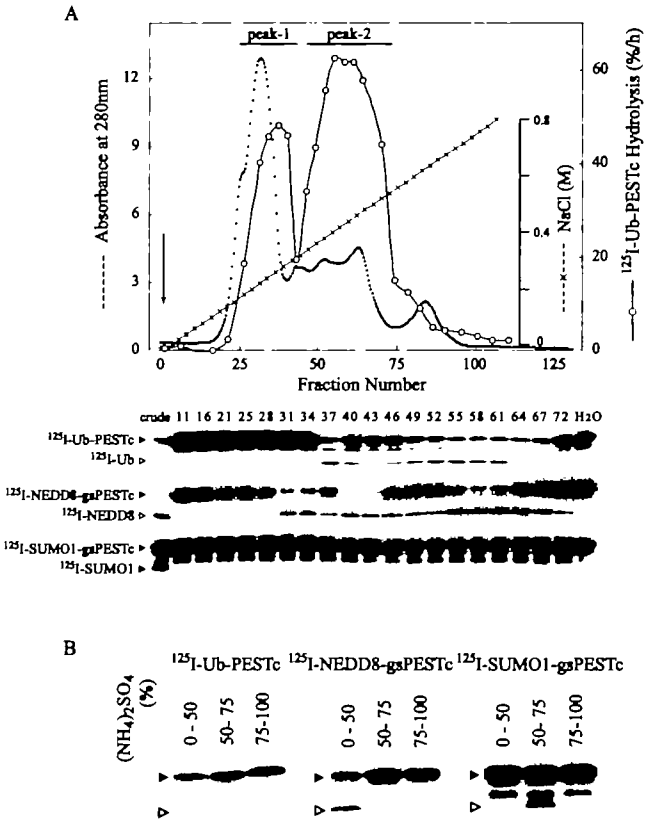


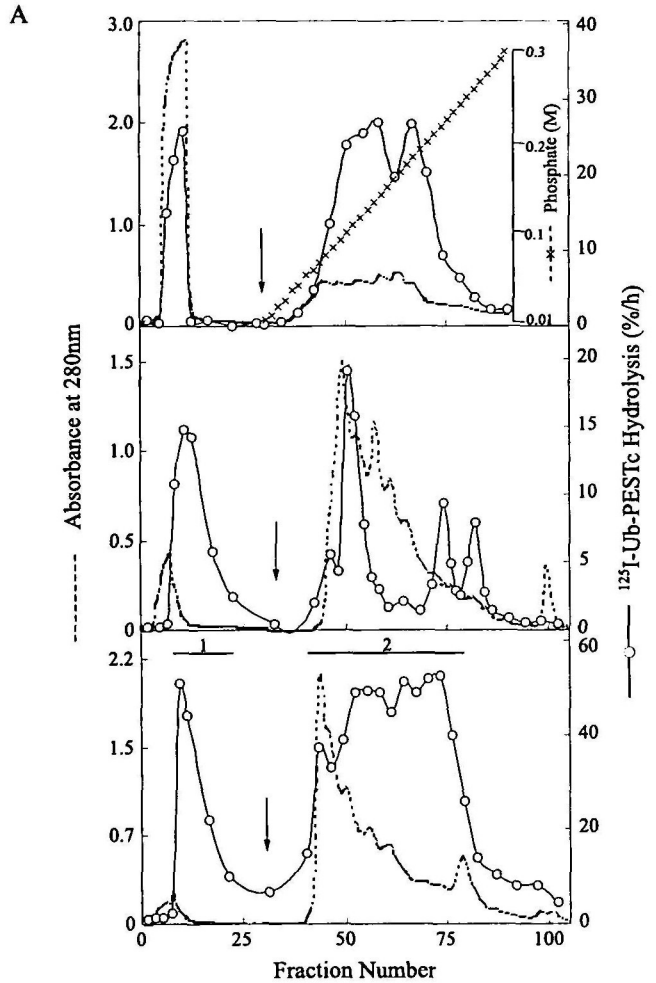
Fig. 1. **Identification of Ub-PESTc, NEDD8-gsPESTc, and SUMO1-gsPESTc degrading enzymes in bovine brain.** (A) Soluble fractions of bovine brain (upper panel) were separated by Q-Sepharose chromatography as described in the text. Fractions of 4 ml were collected, and aliquots (20 μl) of them were assayed as to their ability to hydrolyze ^{125}I-Ub-PESTc as described under "MATERIALS AND METHODS." The fractions under the bars, denoted as peak-1 and peak-2, were pooled and used for further analysis. The vertical arrow indicates the beginning of the salt gradient. The cleavage of ^{125}I-Ub-PESTc, ^{125}I-NEDD8-gsPESTc, and ^{125}I-SUMO1-gsPESTc was also determined by SDS-PAGE followed by autoradiography (lower panel). The numerals above the gels indicate the fraction numbers. The crude and H_2O lanes contained samples incubated in the presence and absence of 5 μg of the crude extract, respectively. (B) The unabsorbed proteins obtained on Q-Sepharose chromatography were fractionated by adding solid $(NH_4)_2SO_4$ to 0-50, 50-75, and 75-100% concentration. The precipitates were dialyzed against Buffer-A and then assayed as to their abilities to cleave ^{125}I-Ub-PESTc, ^{125}I-NEDD8-gsPESTc, and ^{125}I-SUMO1-gsPESTc. The resulting fragments were analyzed by SDS-PAGE followed by autoradiography. The closed and open triangles indicate to where the substrates and their products migrated in the gels, respectively.

recovered in the flow-through fraction (see below). On the other hand, little or no ^{125}I-SUMO1-gsPESTc-degrading activity was detected in any of the column fractions eluted with the salt gradient. However, strong activity against ^{125}I-SUMO1-gsPESTc was also observed in the flow-through fraction.

In order to separate the activities against the Ub- and Ubl-fusion proteins in the flow-through fractions, $(NH_4)_2SO_4$ fractionation was carried out (Fig. 1B). While most of the activities against ^{125}I-Ub-PESTc (left panel) and ^{125}I-NEDD8-gsPESTc (middle panel) were recovered in the 0–50% $(NH_4)_2SO_4$ fraction, the activity against ^{125}I-SUMO1-gsPESTc (right panel) was detected in the 50–75% fraction. Thus, it appears likely that the enzyme(s) responsible for the generation of SUMO1 differs from those releasing Ub and/or NEDD8. The ^{125}I-SUMO1-gsPESTc-degrading activity was recovered as a single peak upon chromatography on an S-Sepharose column (T. Suzuki *et al.*, unpublished data), and its purification is in progress.

For further separation of the enzymes degrading ^{125}I-Ub-PESTc, the 0–50% $(NH_4)_2SO_4$ fraction (Fig. 2A, top panel), and the pooled peak-1 (middle panel) and peak-2 (bottom panel) fractions from the Q-Sepharose column were separately applied to a pre-packed hydroxylapatite column (bed volume, 10 ml) that had been equilibrated with Buffer-B. After washing the column with 5 bed volumes of the buffer, the adsorbed materials were eluted with 120 ml of a linear gradient of 0–0.3 M K-phosphate buffer (pH 6.8). Multiple peaks of ^{125}I-Ub-PESTc-degrading activity were detected for all three enzyme fractions. When each of the activity peaks was chromatographed on a Hitrap Heparin-Sepharose CL-6B column, we could obtain more than 15 distinct peaks of activity against ^{125}I-Ub-PESTc (data not shown).

Among the three enzyme preparations obtained from the Q-Sepharose column (see Fig. 1, A and B, left panel), peak-2 showed the highest activity against ^{125}I-Ub-PESTc and could be separated largely into two fractions on hydroxylapatite chromatography (see Fig. 2A, bottom panel): the flow-through and adsorbed fractions. Therefore, each of these fractions was subjected to affinity chromatography at room temperature on an Ub-Sepharose column that had been equilibrated with 50 mM Tris-HCl (pH 7.2) containing 0.2 mM DTT, 0.1 mM EDTA, and 5% glycerol



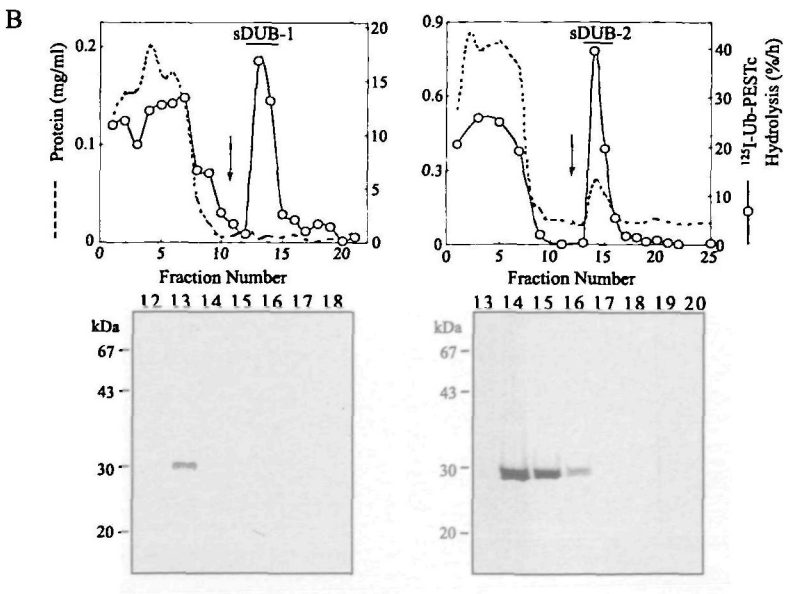


Fig. 2. **Purification of Ub-PESTc degrading enzymes from the soluble fraction of bovine brain.** (A) Aliquots (80 mg each) of the 0–50% $(NH_4)_2SO_4$ fraction (top panel) and the pooled peak-1 (middle panel) and peak-2 (bottom panel) fractions obtained from the Q-Sepharose column (see Fig. 1) were chromatographed on hydroxylapatite columns as described in the text. Fractions of 5 and 2 ml were collected for the flow-through and gradient fractions, respectively, and aliquots (20 μl) of them were assayed as to the abilities to cleave ^{125}I-Ub-PESTc. The fractions under the *bars* in the bottom panel were pooled and used for Ub-Sepharose chromatography. The other symbols are as for (A). (B) The flow-through (2.27 mg) (left panel) and gradient fractions (61.2 mg) (right panel) obtained from the hydroxylapatite column were applied to an Ub-Sepharose column. Fractions of 1.5 ml and 1 ml were collected for the flow-through and gradient fractions, respectively, and aliquots (20 μl) of them were assayed as to the ability to cleave ^{125}I-Ub-PESTc. The fractions under the bars were also subjected to SDS-PAGE followed by staining with Coomassie Blue R-250 (lower panels). The numerals at the top of the gels indicate the fraction numbers.

(Fig. 2B, left and right panels). Although a large portion of the ^{125}I-Ub-PESTc-degrading activity did not bind to the column, we could obtain a single peak of the activity for each fraction upon elution with 50 mM Tris-HCl (pH 9.0) containing 10 mM DTT, 0.1 mM EDTA, and 5% glycerol (Fig. 2B, upper panels). SDS-PAGE of the column fractions with high activity revealed that each of the activity peaks contains a single protein of about 30 kDa (lower panels), indicating that the enzymes were purified to complete or near homogeneity. These Ub-affinity purified enzymes originating from the flow-through and adsorbed fractions from the hydroxylapatite column were tentatively named sDUB-1 and sDUB-2, respectively. Using the same approach, we also attempted to purify the ^{125}I-Ub-PESTc-degrading activities in other enzyme fractions from the Q-Sepharose column [*i.e.*, peak-1 and the 0-50% $(NH_4)_2$-

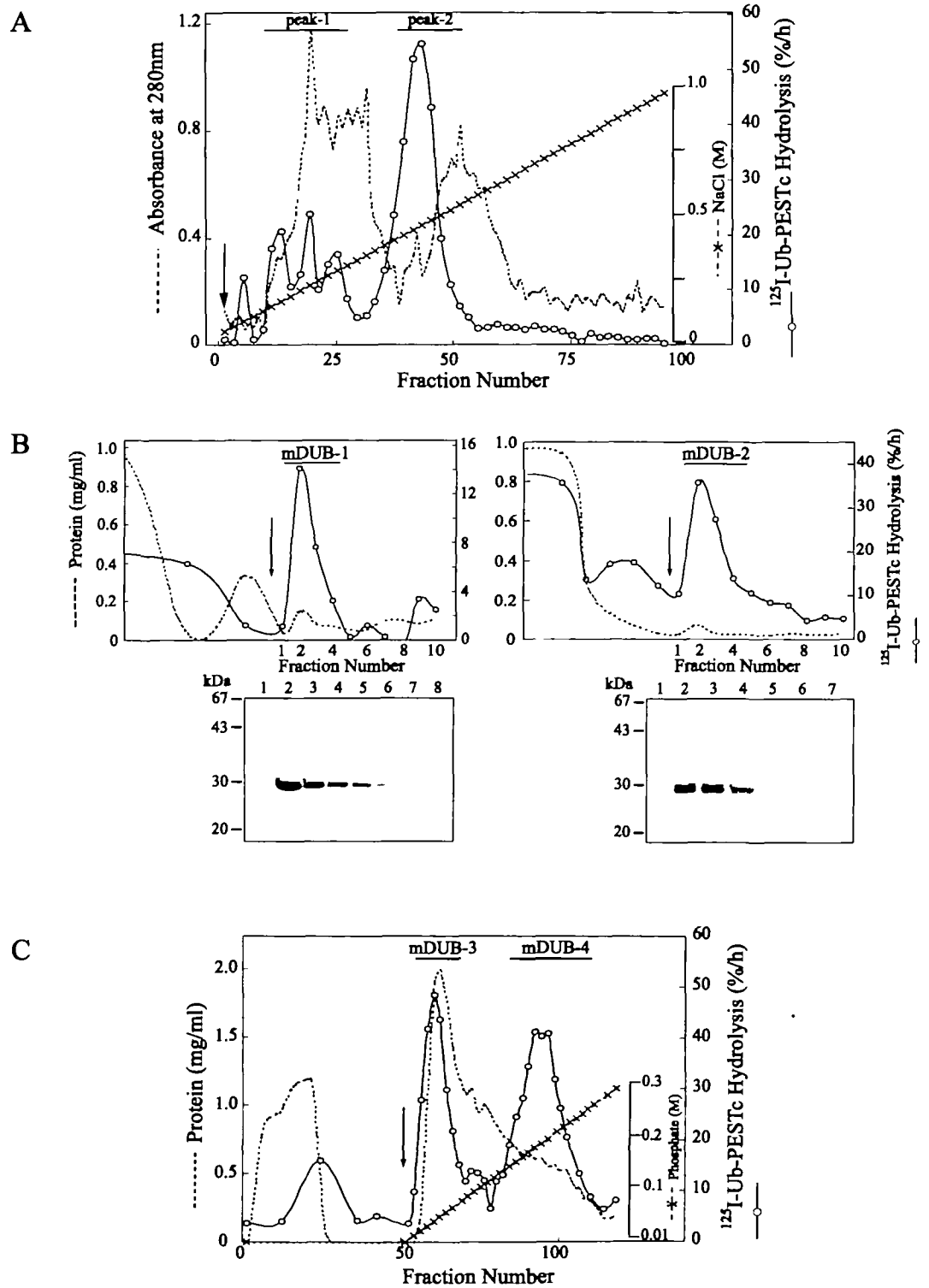


Fig. 3.

SO_4 fraction]. However, little or no activity was adsorbed to the Ub-Sepharose column (data not shown).

Identification and Purification of Membrane-Associated Deubiquitinylating Enzymes—Surprisingly, we found considerably high activity against ^{125}I-Ub-PESTc in the sedimenting fraction obtained by on first low-speed centrifugation of bovine brain homogenates (see above). This activity was consistently recovered in the particulate fraction even after repeated washing, homogenization, and passage through a French Press. Also, it could not be solubilized upon treatment with 1 M NaCl or 1% (v/v) NP-40 (data not shown). However, we were able to solubilize approximately 11 and 40% of the total activity and proteins, respectively, upon treatment of the sedimenting materials with both 5% *n*-heptyl-β-D-thioglucoside and 1% NP-40. Therefore, the solubilized proteins (2.69 g) were subjected to chromatography on a Q-Sepharose column as described in Fig. 1, except that 1% NP-40 was added to Buffer-A. Most of the ^{125}I-Ub-PESTc-degrading activities were adsorbed to the resin, and could be separated into multiple peaks upon elution with a linear gradient of 0–0.8 M NaCl (Fig. 3A).

The fractions comprising the two major peaks (indicated by *bar-1* and *bar-2*) were pooled (300 and 106 mg, respectively) and subjected to Ub-Sepharose affinity chromatography as described in Fig. 2B, but using Buffer-A containing 1% NP-40. A large portion of the ^{125}I-Ub-PESTc-degrading activity did not bind to the column, but a single peak of the activity was eluted on washing with 50 mM Tris-HCl (pH 9.0) containing 10 mM DTT, 0.1 mM EDTA, 5% glycerol, and 1% NP-40 (Fig. 3B, upper panels). SDS-PAGE of the column fractions with high activity revealed that the activity peak contains a single protein of about 30 kDa (lower panels) indicating that the enzymes were purified to apparent homogeneity. The Ub-affinity purified membrane-bound 30-kDa enzymes originating from the peak-1 and peak-2 fractions from the Q-Sepharose column (Fig. 3A) were provisionally named mDUB-1 and mDUB-2, respectively.

In order to separate the ^{125}I-Ub-PESTc-degrading activities in the flow-through fractions from the Ub-Sepharose column (see Fig. 3B, right panel), the pooled proteins were applied to a hydroxylapatite column. After collecting the flow-through fractions, proteins bound to the column were eluted with a linear gradient of 10–300 mM phosphate. As shown in Fig. 3C, the ^{125}I-Ub-PESTc-degrading activities adsorbed to the column could be separated into two major peaks, which were called tentatively mDUB-3 and mDUB-4 according to the order of their elution. Each of them

Fig. 3. **Purification of the ^{125}I-Ub-PESTc-degrading enzymes in the particulate fraction of bovine brain.** (A) Proteins (2.69 g) solubilized with 5% *n*-heptyl-β-D-thioglucoside and 1% NP-40 (see text) were separated by Q-Sepharose chromatography as described in Fig. 1A, except that the column buffer also contained 1% NP-40. Fractions of 9 ml were collected, and aliquots (20 μl) of them were assayed as to the abilities to cleave ^{125}I-Ub-PESTc. The fractions under the bars, denoted as peak-1 and peak-2, were pooled and used for further analysis. (B) The fractions under the bars, peak-1 (300 mg, left panel) and peak-2 (106 mg, right panel), obtained from the Q-Sepharose column were pooled and chromatographed on an Ub-Sepharose column as described in Fig. 2B. After collecting the unbound proteins, the column was washed with Buffer-A containing 1% NP-40. The proteins bound to the column were then eluted as described in the text. Aliquots of the fractions under the bar were subjected to SDS-PAGE followed by staining with Coomassie Blue R-250 (lower panels). The numbers at the top of the gels indicate the fraction numbers. (C) The proteins (105 mg) recovered in the flow-through fractions from the Ub-Sepharose column (right panel in B) were pooled and chromatographed on a hydroxylapatite column. Fractions of 5 ml and 1.5 ml were collected for the flow-through and gradient fractions, respectively, and aliquots (20 μl) of them were assayed as to the abilities to cleave ^{125}I-Ub-PESTc. Two major activity peaks eluted with the phosphate gradient were named mDUB-3 and mDUB-4 in the order of their elution.

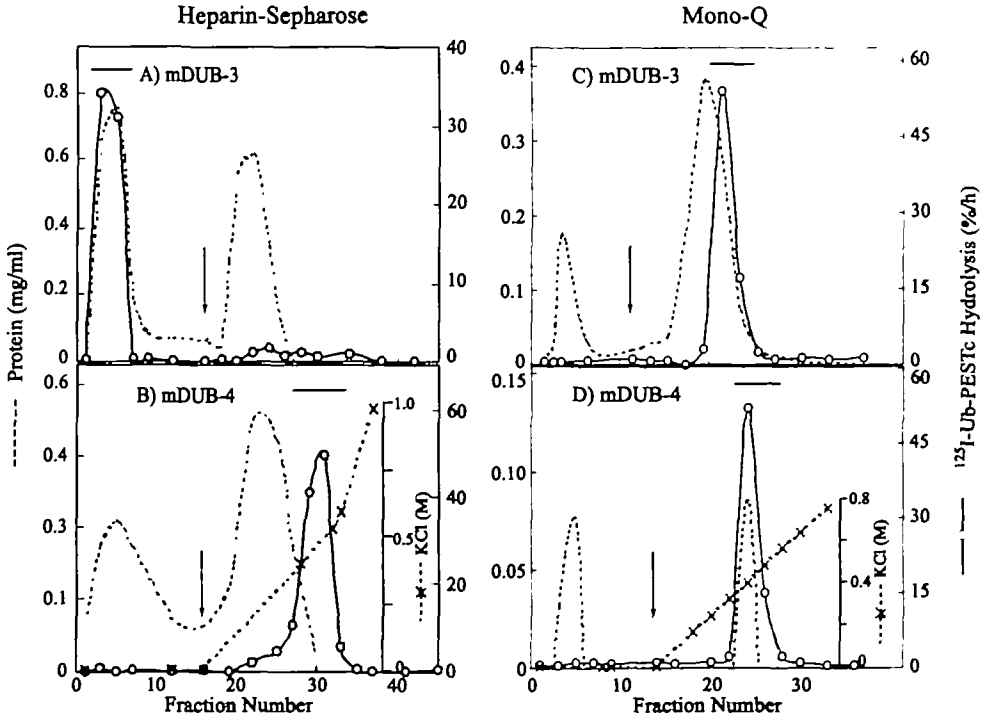


Fig. 4. **Chromatography of mDUB-3 and mDUB-4 on heparin-Sepharose and Mono-Q columns.** The pooled mDUB-3 (25.2 mg) (A) and mDUB-4 (19.1 mg) fractions (B) obtained on hydroxylapatite chromatography (Fig. 3C) were subjected to heparin-Sepharose chromatography. Aliquots (20 μl) of the resulting fractions were assayed as to the ability to cleave ^{125}I-Ub-PESTc. The fractions under the bars for mDUB-3 (22.0 mg) (C) and mDUB-3 (2.64 mg) (D) from the heparin-Sepharose column were further separated by chromatography on a Mono-Q column. The ^{125}I-Ub-PESTc-degrading activities in the resulting fractions were monitored as above. The symbols are as for Fig. 2A.

(mDUB-3, 25.2 mg; mDUB-4, 19.1 mg) was then chromatographed on a Hitrap-Heparin-Sepharose CL-6B column. Figure 4 shows that mDUB-3 exhibits no affinity to the resin (panel A), whereas mDUB-4 bound to the column can be eluted with 0.3 M KCl (panel B). The fractions under the bars (mDUB-3, 22.0 mg; mDUB-4, 2.64 mg) were pooled and again chromatographed on a Mono-Q column. Both mDUB-3 (panel C) and mDUB-4 (panel D) were eluted from the column at approximately the same KCl concentration. These partially purified enzymes (mDUB-3, 0.62 mg; mDUB-4, 0.69 mg) were used for characterization of their properties without further purification.

Structural Relationships among the Isolated sDUBs and mDUBs—To date, 3 UCH family proteins in mammals, called UCH-L1, UCH-L2 and UCH-L3, have been described. They were initially identified on chromatographic separation of bovine brain extracts (*22, 23*), and later the complete primary structures of human UCH-L1 and UCH-L3 (called hUCH-L1 and hUCH-L3, respectively) were determined by cloning their cDNAs (*24, 25*). From the deduced amino acid sequences, the molecular mass of both enzymes has been estimated to be about 30 kDa. In the present studies, we purified 4 putative UCH family enzymes with a size of 30 kDa, named sDUB-1, sDUB-2, mDUB-1, and mDUB-2. Since they all are similar in size to the previously isolated UCHs, it is of necessity to clarify the structural relationships between the enzymes. Moreover, the bovine brain is known to contain a high amount of UCH-L1, which is also called PGP 9.5 (*25*). Therefore, we first examined whether or not any of the bovine brain sDUBs and/or mDUBs is related with PGP 9.5/UCH-L1 by immunoblot analysis using an antibody raised against the latter protein. As shown in Fig. 5A, the antibody reacted much more strongly with sDUB-2 than with sDUB-1. It also reacted with mDUB-2, but very weakly even in comparison with sDUB-1. However, no significant reactivity was observed with mDUB-1. The PGP 9.5/UCH-L1 antibody reacted neither with the partially purified mDUB-3 nor mDUB-4. This result together with the finding of a relatively high abundance of sDUB-2 in the brain strongly suggests that the enzyme is identical to PGP 9.5/UCH-L1. However, all of the purified DUBs appear to be distinct from

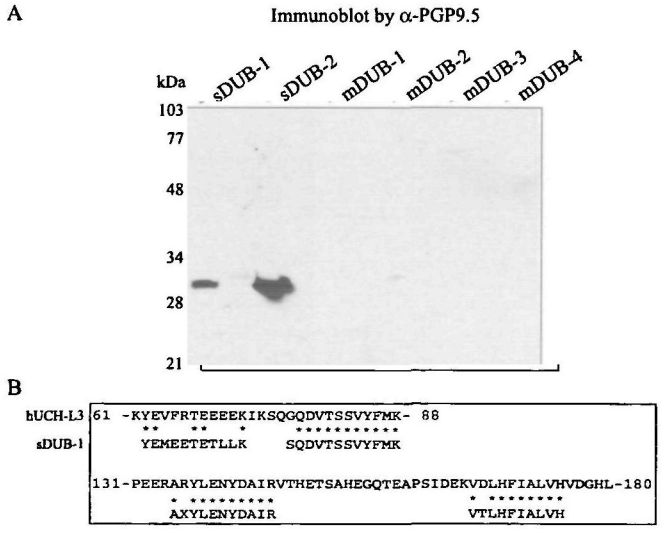


Fig. 5. **Immunological and biochemical analyses of sDUBs and mDUBs.** (A) The same amount (0.2 μg) of the purified sDUB-1, sDUB-2, mDUB-1, and mDUB-2, and the partially-purified mDUB-3 (2.0 μg) and mDUB-4 (1.0 μg) were subjected to SDS-PAGE, followed by immunoblot analysis using a human anti-PGP 9.5/UCH-L1 antibody. (B) The amino acid sequence of the internal fragment of sDUB-1 was determined by Edman degradation as described under "MATERIALS AND METHODS," and compared with that of human UCH-L3 (*24*). Amino acid residues are numbered from the N-terminus. Asterisks show identical amino acids.

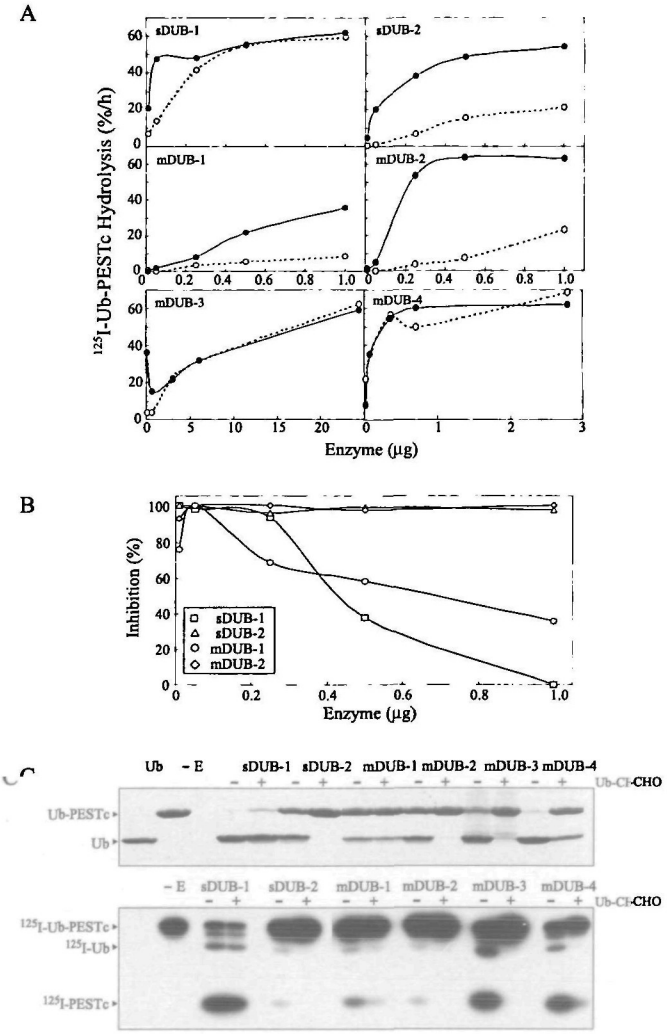


Fig. 6. **Effects of poly-L-lysine and Ub-CHO on the hydrolysis of Ub-PESTc by sDUBs and mDUBs.** (A) Increasing amounts of the purified sDUB-1, sDUB-2, mDUB-1, and mDUB-2, and the partially-purified mDUB-3 and mDUB-4 were incubated for 1 h with 1 μg of ^{125}I-Ub-PESTc in the presence (●) and absence (○) of poly-L-Lys (10 μg/ml). After the incubation, the radioactivity released into acid-soluble products was determined. (B) Increasing amounts of the purified DUBs were incubated with Ub-CHO (1 μM) for 10 min at 37°C. The samples were then further incubated for a further next 1 h with 1 μg of ^{125}I-Ub-PESTc. The inhibitory effect of Ub-CHO was expressed as a percentage of the activity of each enzyme without the inhibitor. (C) The enzymes (0.5 μg) were incubated in the absence (−) and presence (+) of 1 μM Ub-CHO as in (B). The resulting samples were further incubated for a further 1 h with ^{125}I-Ub-PESTc. They were then subjected to SDS-PAGE followed by staining with Coomassie Blue R-250 (upper panel) or autoradiography (lower panel).

each other, since their immunoreactivities to the anti-PGP 9.5/UCH-L1 antibody are markedly different.

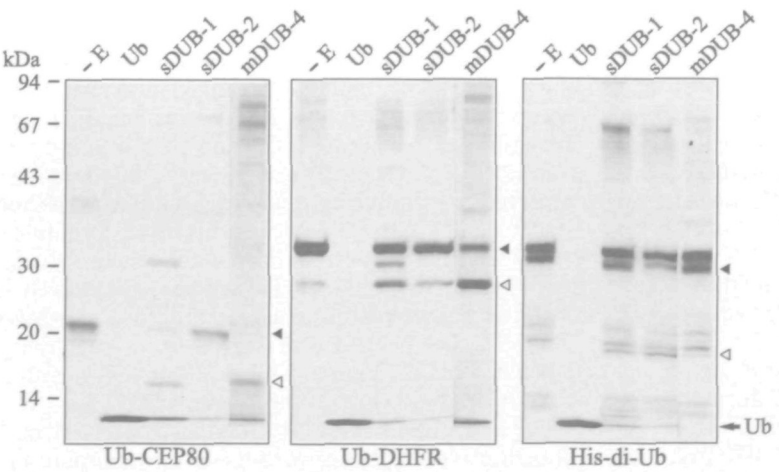
It has been reported that the primary structure of UCH-L1 exhibits high similarity to that of UCH-L3 and to the partial sequence of UCH-L2 (*24*). In addition, the present study revealed that the anti-PGP 9.5/UCH-L1 antibody also reacted with sDUB-1, although to much lesser extent than with sDUB-2. Therefore, we examined whether or not sDUB-1 may correspond to UCH-L2 or UCH-L3. For this, we performed partial sequencing analysis of several internal fragments of sDUB-1. As shown in Fig. 5B, the amino acid sequence of the fragments of sDUB-1 exhibited high similarity to that of hUCH-L3. Therefore, we concluded that sDUB-1 is a bovine homolog of human UCH-L3, and that the difference of several residues between them may be due to the species difference. In addition, the considerable reactivity of sDUB-1 to the anti-PGP 9.5/UCH-L1 antibody (Fig. 5A) might be due to the high similarity between UCH-L1 and UCH-L3, amino acid identity being 52%. Of note is the finding that the sequence of an internal fragment of sDUB-1, VTLHFIA, overlaps that of the previously reported bovine UCH-L2 (NNLRFIA) (*24*). However, the sequences of these two fragments are not identical, indicating that bovine sDUB-1 (equivalent to UCH-L3) and UCH-L2 are different enzymes. Interestingly, partial sequencing analysis of several internal fragments of mDUB-1 and mDUB-2 revealed their high similarities to UCH-L1 and UCH-L3, indicating that they are members of a family of UCH enzymes.

Enzymatic Properties of sDUBs and mDUBs—The activities of all 6 DUBs isolated in the present study were completely inhibited by 1 mM NEM (data not shown), indicating that they belong to a family of Cys proteases, as reported for other DUBs (*10, 23*). We also examined the effect of poly-L-Lys on the enzyme activities, since this poly-cationic agent is known to markedly stimulate most UCHs isolated from chick skeletal muscle (*11*). As shown in Fig. 6A, the activities of all of the purified sDUB-1, sDUB-2, mDUB-1, and mDUB-2 were markedly stimulated by treatment with 10 μmg/ml of poly-L-Lys. However, it is noteworthy that the stimulatory effect of the agent on the activity of sDUB-1 could only be seen in the presence of a low concentration of the enzyme (*i.e.*, below 0.1 μM, which

A

Substrate / Enzyme	^{125}I-NEDD8 -gsPESTc	^{125}I-SUMO1 -gsPESTc	Ub-CEP80	Ub-DHFR	His-di-Ub
sDUB-1	+	-	+	+	+
sDUB-2	-	-	+	+	+
mDUB-1	+	-	+	-	±
mDUB-2	+	-	+	-	±
mDUB-3	-	-	+	-	+
mDUB-4	+	-	+	+	±

B



C

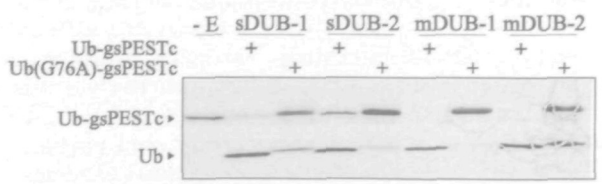


Fig. 7. **Hydrolysis of ^{125}I-NEDD8-gsPESTc, ^{125}I-SUMO1-gsPESTc, Ub(G76A)-PESTc, and various Ub-extension proteins by sDUBs and mDUBs.** (A) The purified sDUB-1, sDUB-2, mDUB-1, and mDUB-2 (0.5 μg), and the partially purified mDUB-3 (6.1 μg) and mDUB-4 (0.7 μg) were incubated to assay their abilities to convert 1 μg of ^{125}I-NEDD8-gsPESTc and ^{125}I-SUMO1-gsPESTc into radioactive acid-soluble products. They were also incubated with 2 μg of Ub- CEP80, Ub-DHFR, and His-di-Ub. After the incubation, the samples were subjected to SDS-PAGE followed by staining with Coomassie Blue R-250. When more than 5% or below 1% of the substrates was degraded during the incubation, their susceptibility to the enzymes was expressed as "+" or "−", respectively. (B) As examples, the cleavage patterns of Ub-CEP80, Ub-DHFR, and His-di-Ub on the Coomassie-stained SDS-PAGE gels in (A) are shown for those incubated with sDUB-1, sDUB-2, and mDUB-4. The closed and open triangles indicate where the substrates and their products migrated to in the gels, respectively. (C) Cleavage of Ub(G76A)-gsPESTc by the purified sDUB-1, sDUB-2, mDUB-1, and mDUB-2 was measured as described in Fig. 6C, except that Ub-gsPESTc and Ub(G76A)-gsPESTc were used as substrates. The reaction products were then subjected to SDS-PAGE followed by staining with Coomassie Blue R-250.

corresponds to 0.3 μg). It is also of note that the specific activity of sDUB-1 is the highest among the purified DUBs. In contrast, poly-L-Lys showed little or no effect on the activity of mDUB-3 or mDUB-4, except when its effect was examined with low concentrations of mDUB-3.

To determine the effect of Ub-aldehyde (Ub-CHO), the purified sDUBs and mDUBs were incubated with ^{125}I-Ub-PESTc in the absence and presence of the reagent. As shown in Fig. 6B, Ub-CHO at 1 μM almost completely blocked the activity of sDUB-2 and mDUB-2 at all enzyme concentrations tested. It also strongly inhibited the activities of sDUB-1 and mDUB-1 at low concentrations of the enzymes (*e.g.*, below 0.1 μM) but not with high concentrations (*e.g.*, at 0.33 μM). These differential inhibitory effects of Ub-CHO strongly suggest that sDUB-1 and mDUB-1 are distinct from sDUB-2 and mDUB-2, respectively. However, it remains unclear why sDUB-1 and mDUB-1 are much less sensitive to Ub-CHO than sDUB-2 and mDUB-2. In fact, it has been reported that the deubiquitinylating activity of the 26S proteasome is not sensitive to Ub-CHO (*26*).

To confirm the effect of Ub-CHO, we incubated each of the purified sDUBs and mDUBs with unlabeled Ub-PESTc or ^{125}I-labeled Ub-PESTc in the presence and absence of Ub-CHO. The reaction products were then subjected to SDS-PAGE followed by staining with Coomassie Blue R-250 or by direct autoradiography of the gels (Fig. 6C, upper and lower panels, respectively). All 4 DUBs were found to precisely cleave the substrate into Ub and PESTc. The effect of Ub-CHO was essentially the same as that observed with high concentrations of the enzymes (Fig. 6B). In order to also confirm whether the partially purified mDUB-3 and mDUB-4 show deubiquitinylating activities, we performed electrophoretic analysis as above. Both enzymes precisely cleaved the substrates into Ub and PESTc (Fig. 6C). Moreover, Ub-CHO strongly inhibited both the enzyme activities. These results indicate that mDUB-3 and mDUB-4 also belong to a family of deubiquitinylating enzymes.

Substrate Specificity of the sDUBs and mDUBs—We then examined whether or not the isolated sDUBs and mDUBs are also capable of generating free Ub from NEDD8-gsPESTc and SUMO1-gsPESTc, and various Ub-extension proteins, such as Ub-CEP80, Ub-DHFR, and His-Di-Ub. As summarized in Fig. 7 (A and B), none of the DUBs could release SUMO1 from ^{125}I-SUMO1-gsPESTc. Weak activity as to the hydrolysis of ^{125}I-NEDD8-gsPESTc was observed upon incubation with sDUB-1, mDUB-1, and mDUB-2. On the other hand, all of the enzymes could generate free Ub from Ub-CEP80 and His-di-Ub, although cleavage of the latter substrate was much less significant with mDUB-1, mDUB-2, and mDUB-4. In addition, mDUB-1, mDUB-2, and mDUB-3 showed little or no activity against Ub-DHFR, unlike sDUB-1, sDUB-2, and mDUB-4. Judging from these results, we conclude that all the six DUBs isolated in the present study are functionally different from each other.

Next, we measured the cleavage of Ub(G76A)-gsPESTc, in which Gly at position 76 of the C-terminal end of Ub was replaced by Ala, by the purified sDUB-1, sDUB-2, mDUB-1, and mDUB-2. As shown in Fig. 7C, the degrading activity against Ub(G76A)-gsPESTc of these purified enzymes almost completely disappeared, except that considerable activity was observed for mDUB-2, indicating that these enzymes presumably recognize the Gly residue at the C-terminal end of the Ub moiety for cleavage. However, this recognition does not seem to be strict, because degrading activity against Ub(G76A)-gsPESTc was detected for all enzymes, nevertheless sDUB-1 and sDUB-2 are thought to be identical to well-characterized UCH-L3 and UCH-L1/PGP 9.5, respectively.

DISCUSSION

In the present study, we found the presence of an unusually large number of DUBs in both soluble and membranous fractions of bovine brain. However, an even larger number of related enzymes may exist in cells, since other enzymes may also exist that are unable to degrade Ub-PESTc, a substrate that we used in this work. It is conceivable that the expanding family of bovine brain DUBs has a Cys active site, since they are sensitive to inhibition by sulfhydryl-blocking reagents, such as NEM, like other known DUBs. All eukaryotes contain DUBs encoded by at least two gene families that are structurally unrelated: the UCH (Ub C-terminal hydrolase) family and the UBP (Ub-specific protease) family (*2, 5, 10*). These two UBP- and UCH-family proteins are sensitive to inhibition by Ub-CHO, a specific inhibitor of DUBs (*5, 13*). Most of the Ub-PESTc-cleaving activities separated by chromatographic procedures in the present study were strongly inhibited by Ub-CHO (data not shown), although some of the purified DUBs, such as sDUB-1 and mDUB-1, differed considerably in their sensitivity to Ub-CHO depending on the enzyme concentration (see Fig. 6B). Furthermore, they precisely cleaved the peptide-bond between Ub and the PESTc peptide in Ub-PESTc, as judged on SDS-PAGE analysis. Thus, Ub-PESTc should be an excellent substrate for the assaying of cellular DUBs (*11*).

We have purified two bovine brain cytosolic enzymes, called sDUB-1 and sDUB-2, by affinity chromatography using Ub as a ligand (Fig. 2B). Of them, sDUB-2 was found to be identical to PGP 9.5/UCH-L1, an abundant protein in the brain, upon immunoblot analysis using an antibody against human PGP 9.5 (Fig. 5A). Intriguingly, the primary structures of bovine UCH-L1 and UCH-L3, both of which also have a molecular mass of about 30 kDa, have been reported to exhibit a high sequence similarity, although the structure of UCH-L2, which is distributed ubiquitously in many tissues, has not yet been determined (*24*). UCH-L3 is a hematopoietic cell specific isoform, whereas UCH-L1 is found at high levels in neural and diffuse neuroendocrine cells (*24*). Our sequence analysis data revealed that sDUB-1 is a homolog of human UCL-L3, but that it differs from bovine UCH-L2 (Fig. 5B and see text).

Of particular interest is the finding that a large number of presumptive DUBs are present in the sedimenting materials obtained on low-speed centrifugation of brain homogenates. However, we could solubilize only about 11% of the total ^{125}I-Ub-PESTc-degrading activity in the homogenates by treatment with 5% *n*-heptyl-β-D-thioglucoside and 1% NP-40, indicating that more tightly membrane-associated DUBs must exist. Nevertheless, we were able to purify two novel membrane-bound enzymes, mDUB-1 and mDUB-2, to apparent homogeneity, and partially purify two other presumptive membranous enzymes, called

mDUB-3 and mDUB-4, from the same solubilized fractions (Figs. 3 and 4).

Although mDUB-1 and mDUB-2 have a similar size of about 30 kDa, they appear to be different from each other as well as from sDUB-1 and sDUB-2, that also have a size of about 30 kDa, in a number of criteria. (i) While sDUB-1, sDUB-2, and mDUB-2 reacted with a human PGP 9.5 antibody to markedly different extents, mDUB-1 did not react with it at all (Fig. 5A). (ii) The stimulatory effect of poly-L-Lys on the Ub-PESTc-cleaving activities of the enzymes was significantly different (Fig. 6A), although it is totally unknown at present how poly-L-Lys dramatically activates them. (iii) While Ub-CHO at 1 μM completely inhibited the activities of sDUB-2 and mDUB-2 against Ub-PESTc with all enzyme concentrations tested, its effect on sDUB-1 and mDUB-1 was dependent on the enzyme concentration (Fig. 6, B and C). (iv) While sDUB-1 and sDUB-2 were capable of releasing Ub from Ub-DHFR, neither mDUB-1 nor mDUB-2 could (Fig. 7, A and B). (v) The degradation of Ub(G76A)-gsPESTc by the purified sDUB-1, sDUB-2, mDUB-1, and mDUB-2 was quite low, compared to that of unmodified Ub-gsPESTc, although mDUB-2 showed significant activity (Fig. 7C). Thus, it seems clear that each of the purified sDUB-1, sDUB-2, mDUB-1, and mDUB-2 differs from the others and represents an authentic deubiquitinylating enzyme. The partially purified mDUB-3 and mDUB-4 also appear to be deubiquitinylating enzymes, as judged from their differential sensitivity to Ub-CHO, and their ability to process Ub-PESTc precisely into Ub and PESTc (Fig. 6). In addition, these membrane-bound enzymes appear distinct from each other as well as from the purified mDUB-1 and mDUB-2, since their chromatographic behavior on Ub-Sepharose and heparin-Sepharose columns differs markedly (see text).

The presence of a large number of soluble and membranous DUBs in the bovine brain may contribute to the reversal of the ubiquitinylation reaction catalyzed by the Ub-ligating system as well as to the production of free Ub from its precursors. Namely, the role of DUBs has been implicated in the production of a functional Ub moiety from poly-Ub and Ub-fused gene products, "proofreading" of incorrectly ubiquitinylated proteins, "trimming" of abnormal poly-Ub structures, release of the Ub moiety from a free poly-Ub chain, or disassembly of degradation intermediates generated by the proteasome (reviewed in Refs. *2* and *10*). Accumulating evidence has also indicated that deubiquitinylation from poly-ubiquitinylated proteins plays important roles in a variety of physiological processes. For example, the product of the fat facets genes (*faf*) in *Drosophila* is required for eye facet development (*27*). Recently, Fam, a mammalian homologue Faf, was shown to catalyze the removal of the Ub moiety from ubiquitinylated AF-6, which is known to serve as one of the peripheral components responsible for cell-cell adhesion and to function in the downstream of the Ras signaling pathway (*28*). A neural UCH is important for long-term facilitation in *Aplysia* (*29*). Yeast UBP3, a 110 kDa SIR4-binding protein, functions as an inhibitor of the silencing of transcription (*30*). HAUSP (*h*erpesvirus-*a*ssociated *U*b-specific *p*rotease), a 135 kDa UBP, is dynamically associated with PML nuclear bodies and herpesvirus protein Vmw-110, suggesting its involvement in the control of viral gene expression (*31*). It has been reported that two mouse DUBs are immediate early gene products induced by cytokines. Mouse DUB-1 is induced by IL-3 (*32*) and DUB-2 is induced by IL-2 (*33*), and intriguingly DUB-1 inhibits cell growth when overexpressed. Moreover, the human *tre-2* oncogene encodes the N-terminal part of a DUB similar to yeast Doa4, indicating a role for the Ub system in mammalian growth control (*6, 34*). The recently identified UBPY accumulates upon growth stimulation and its levels decrease in response to growth arrest induced by cell-cell contact, suggesting that it is stringently correlated with cell proliferation and plays a role in regulating the overall function of the Ub-proteasome pathway (*35*). In contrast, a nuclear localized UCH, named BAP1, is suggested to be a new tumor suppresser gene (*36*). It was very recently reported that certain patients with Parkinson's desease have a unique Ile93Met mutation in their UCH-L1 gene (*37*). However, it remains to be explained how these DUBs are responsible for these phenotypes, although it is notable that a loss of function of these genes through deletion or a mutation or a gain of function through their overexpression causes the induction of the abnormalities given.

There is accumulating evidence that various Ubls are present universally in a wide variety of eukaryotic cells (*38-41*). Of these, SUMO1 (yeast Smt3 homolog) is covalently ligated to cytosolic protein RanGAP1 to associate with the nuclear pore complex or nuclear proteins, such as PML and Vmn110 (*36, 39*), presumably through a novel pathway related to the Ub-ligating system, being activated by a heterodimeric complex and conjugated by Ubc9 (reviewed in Refs. *40* and *41*). The gene coding NEDD8 was first identified as one of the multiple *n*eural precursor cell-*e*xpressed *d*evelopmentally *d*own-regulated genes (*42*). Recently, Rub1 and NEDD8 were found to be ligated to yeast Cdc53 and mammalian cullin-4A, respectively, which are members of the family of human cullin/Cdc53 proteins functioning as essential components of a multifunctional Ub-protein ligase E3 complex (*43*). This modification also requires a new pathway related to the Ub-ligating system, consisting of a heterodimeric complex and Ubc12 as the E1- and E2-like enzymes, respectively (*44, 45*). However, the role of the covalent modification of the target proteins by the Ubls is largely unknown.

Interestingly, most Ubls, including SUMO1, Smt3, NEDD8, and Rub1, are synthesized as proproteins carrying C-terminal extension peptides. Therefore, the cleavage of the extra-peptides to expose the C-terminal Gly residue, like Ub, should be essential for their ligation to target proteins through an isopeptide linkage. Moreover, it has been reported that Sentrin/SUMO1 is processed to generate the C-terminal Gly residue prior to its conjugation to appropriate target protein(s) (*46*). A similar observation has been reported for the necessity of processing of NEDD8 in the covalent modification of proteins (*47*). Taken together, it is conceivable that Ubls are processed prior to ligation with target proteins. However, the enzymes catalyzing the maturation of their precursor proteins in mammalian tissues have not been reported so far.

In the present study, we showed that soluble extracts of bovine brains give multiple peaks of activity capable of releasing NEDD8 from ^{125}I-NEDD8-gsPESTc upon chromatography on a Q-Sepharose column. Interestingly, these activity peaks largely overlapped those of the activity

against ^{125}I-Ub-PESTc (Fig. 1). Furthermore, the purified sDUB-1, mDUB-1, and mDUB-2 could cleave off the PESTc peptide from both ^{125}I-Ub-PESTc and ^{125}I-NEDD8-gsPESTc (Fig. 7, A and B). Therefore, it appears that some deubiquitinylating enzymes may be responsible for the processing of both Ub and NEDD8 precursors. In fact, it was recently reported that UCH-L3, a homolog of sDUB-1, is capable of cleaving the C-terminus of NEDD8 *in vitro* in addition to certain Ub precursors (*48*). On the other hand, we found that the ^{125}I-SUMO1-gsPESTc degrading activity could clearly be separated from the activities against both ^{125}I-Ub-PESTc and ^{125}I-NEDD8-gsPESTc upon $(NH_4)_2SO_4$ fractionation (Fig. 1B). Thus, it also seems likely that specific enzymes exist that are distinct from DUBs, and capable of generating NEDD8 and SUMO1 from their proproteins. Very recently it was reported that a new yeast Smt3/SUMO1-specific protease, called Ulp1, which exhibits Smt3 C-terminal hydrolase activity, is required for cell-cycle progression (*49*), but the relationship between Ulp1 and the presently-described ^{125}I-SUMO1-gsPESTc degrading enzyme is unknown. In order to clarify whether the enzymes identified in the present study are indeed involved in the processing of NEDD8- and SUMO1-precursors or the release of Ubls from their conjugated proteins, purification and characterization of the enzymes are essential, and are in progress.

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