DUB-1A, a Novel Deubiquitinating Enzyme Subfamily Member, Is Polyubiquitinated and Cytokine-inducible in B-lymphocytes*

Kwang-Hyun Baek‡§§, Myung-Sun Kim‡§§, Yong-Soo Kim‡, Ju-Mi Shin‡§, and Hee-Kyung Choi‡

From the ¶Cell and Gene Therapy Research Institute and the §Graduate School of Life Science and Biotechnology, Pochon CHA University, CHA General Hospital, Seoul 135-081, Korea

Received for publication, May 7, 2003, and in revised form, October 6, 2003
Published, JBC Papers in Press, October 28, 2003, DOI 10.1074/jbc.M304774200

Recently, we isolated the Dub-2A gene, which encodes a novel murine deubiquitinating enzyme subfamily member, from a bacterial artificial chromosome library clone by PCR amplification with degenerate PCR primers for the Dub-2 cDNA (Baek, K.-H., Mondoux, M. A., Jaster, R., Fire-Levin E., and D’Andrea, A. D. (2001) Blood 98, 636–642). In this study, we analyzed two more clones from the library to isolate genes encoding other deubiquitinating enzymes. Dub-1A, which encodes the shortest member of the Dub subfamily of deubiquitinating enzymes so far, has been identified in both clones and characterized. Sequence analysis showed that Dub-1A encodes a 468-amino acid protein that has a molecular mass of ~51 kDa and that contains a putative catalytic domain (Cys, His, and Asp) conserved among DUB proteins. The amino acid sequence of Dub-1A is 84.5, 84.7, and 85.3% identical to those of Dub-1, Dub-2, and Dub-2A, respectively. Reverse transcription-PCR revealed that Dub-1A is expressed not only in B-lymphocytes in response to interleukin-3 stimulation, but also in T-lymphocytes, brain, heart, liver, lung, kidney, ovary, and spleen. This suggests that Dub-1A may play essential roles in each of these organs. In vivo and in vitro deubiquitinating enzyme assays showed that Dub-1A has functional deubiquitinating activity and that the 5'-flanking sequence of Dub-1A has a functional enhancer domain as shown in Dub-1 and Dub-2A. Interestingly, immunoblot analysis revealed that Dub-1A is polyubiquitinated, indicating that it is degraded through proteasome-mediated degradation. In the absence of JAK2, Dub-1A was expressed at a lower level. This suggests that DUB-1A functions downstream of JAK2 kinase in the interleukin-3 signaling pathway.

It is evident that a balance between ubiquitination and deubiquitination processes plays a pivotal role in regulating the destiny of proteins within cells. The deviation of balanced ubiquitination and deubiquitination may lead to the aberration of intracellular processes, including cell cycle progression, transcriptional activation, signal transduction, antigen presentation, apoptosis (or programmed cell death), oncogenesis, preimplantation, and DNA repair (1–7). A number of studies have been carried out to investigate ubiquitin conjugation and its role in regulating protein degradation. Ubiquitin, a small polypeptide of 76 amino acids, functions as a tag for proteasome-mediated protein degradation (8–10) and is conjugated to substrates by a series of reactions catalyzed by several classes of enzymes. These include ubiquitin-activating enzymes (E1), ubiquitin carrier proteins (E2), and ubiquitin-protein isopeptide ligases (E3). E1 enzymes form a thiol ester bond with the glycine of ubiquitin in an ATP-dependent process. Then, E2 enzymes receive ubiquitin from the E1 enzymes by a trans-thiolation process. Finally, the ubiquitin with E2 enzymes is transferred to the lysine residue of the protein substrate by E3 enzymes (3, 8, 11, 12). Thus, the ubiquitin system is hierarchical, and the identified numbers of these enzymes are the highest for E3, followed by E2 and E1 (13). A novel ubiquitination factor (E4) is essential for efficient multiubiquitination in yeast (14). Proteins conjugated with multiple units of ubiquitin (Ub) are recognized by the 26S proteasome, where they are degraded to small polypeptides in an ATP-dependent manner (15–19).

Recent investigations demonstrated that deubiquitinating enzymes, which cleave ubiquitin from protein substrates, also regulate protein degradation. This process either rescues proteins from degradation or accelerates protein degradation (20, 21). The deubiquitinating enzymes are cysteine proteases and are classified into two major families: the ubiquitin C-terminal hydrolase family and the ubiquitin-processing protease family (20, 21). According to the Swiss Protein Database, >90 deubiquitinating enzymes identified so far reveal structural diversity, suggesting a broad range of substrate specificity. 23 deubiquitinating enzymes, including the tumor suppressor CYLD1, have recently been identified by chemistry-based functional proteomics in EL4 cells (22). It is expected that with information obtained from genome sequencing projects in various living organisms, including human and mouse, a number of genes encoding a deubiquitinating enzyme will be identified by various methods (23).

Recently, we identified a few members of a novel Dub subfamily in lymphocytes, including Dub-1, Dub-2, and Dub-2A (2, 24, 25). Both Dub-1 and Dub-2 were originally cloned as immediate-early genes and are induced by cytokines in B- and T-lymphocytes, respectively (2, 24). Dub-1 is induced by stimula-

* The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; E4, ubiquitin conjugation factor; Ub, ubiquitin; IL, interleukin; BAC, bacterial artificial chromosome; RT, reverse transcription; HA, hemagglutinin; GST, glutathione S-transferase; WT, wild-type; DN, dominant-negative; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; STAT, signal transducer and activator of transcription.
tion with interleukin (IL)-3, IL-5, and granulocyte/macrophage colony-stimulating factor, whereas Dub-2 is induced by stimulation with IL-2 only (2). In addition, it has been reported that Dub-2 is capable of promoting IL-2-mediated signaling and can suppress apoptosis in lymphocytes after withdrawal of growth factor (24, 26). However, substrates for these Dub enzymes have not been identified yet, even though it has been suggested that Dub-2 may be involved in reversal of Cbl-mediated p85 ubiquitination (24, 27).

In this study, we describe the cloning of both complementary and genomic DNA for a novel deubiquitinating enzyme, Dub-1A, which is classified as one of the Dub subfamily members. The sequences of the putative polypeptide and enhancer element for Dub-1A are highly homologous to those for previously known Dub subfamily members (Dub-1, Dub-2, and Dub-2A) and reveal deubiquitinating enzyme activity in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Screwing of the Bacterial Artificial Chromosome (BAC) Library and Cloning of the Dub-1A Gene—The murine genomic library in the pBluBAC11 vector was screened by genomic PCR with two primers (Dub-1A 5'-GCGGATCCTTTGAGACATCTGCCTTGAAA-3' and Xho5, 5'-ATCTCGAGGTGTCCAGAGGACCTGCTG-3') derived from the sequence of the Dub-2 gene. Using the same primers, genomic PCR products containing the Dub-1A gene from two of three positive BAC clones were generated, cloned, and sequenced.

Cells, Tissues, and Culture Conditions—Ba/F3 cells (IL-3-dependent murine pro-B cells) were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 10 μg murine IL-3 (R&D Systems), except for those used in cytokine dose-dependent Dub-1A induction experiments. CTL-2 cells (IL-2-dependent murine pro-T cells) were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 10 μg murine recombinant IL-2 (R&D Systems). Various tissues (mouse brain, heart, liver, lung, kidney, ovary, and spleen; 129/w) were obtained.

NHiST3 and COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum in a 5% CO₂ atmosphere. NHiST3 cells were transiently transfected with 2 μg of pcDNA3-Myc-Dub-1A or pcDNA3-Myc-Dub-1A (C60S) with pcMT123-HA-ubiquitin using LipofectAMINE according to the manufacturer's instructions (Invitrogen).

RNA Extraction and PCR—Total cellular RNA was isolated from Ba/F3 cells, CTL-2, and various tissues using TRizol (Invitrogen). The tissues used for RNA extraction were from brain, heart, kidney, liver, lung, spleen, ovary, and testis. The RNAs were subjected to reverse transcription (RT-PCR). We performed RT-PCR using a pair of primers designed as Dub-1A-specific based on the Dub-1A sequence. The nucleotide sequences of these primers are as follows: 1A-S1, 5'-ATGTCACCTCCTAGT-3' and 1A-S2, 5'-TACCTTTAGGTC-3'. Degenerate PCR was also performed using a pair of primers designed for Dub enzymes (Dub-1, Dub-2, and Dub-1A). The nucleotide sequences of these primers are as follows: Dub-1A: 5'-GCGGATCCTTTGAGACATCTGCCTTGAAA-3' and Xho5, 5'-ATCTCGAGGTGTCCAGAGGACCTGCTG-3').

In Vivo and in Vitro Deubiquitination Assays—The effect of Dub-1A expression on the ubiquitin/proteasome system was analyzed by transfecting pcDNA3-Myc-Dub-1A or pcDNA3-Myc-Dub-1A (C60S) with pMT123-HA-ubiquitin into NHiST3 cells. After a 24-h transfection, cells were trypsinized and lysed. 10 μg of total proteins were loaded onto each lane of a 10% SDS-polyacrylamide gel for immunoblot analysis using anti-hemagglutinin (HA) antibody (Roche Applied Science). Equal loading was verified by immunoblotting against anti-Myc antibody (Santa Cruz Biotechnology).

In vitro deubiquitination assay of Ub-β-galactosidase fusion protein has been described previously (28). The deubiquitinating activity of Dub-1A was analyzed by immunoblotting with rabbit anti-β-galactosidase antisera (Cappel) and rabbit anti-glutathione S-transferase (GST) antisera (Santa Cruz Biotechnology) using an ECL detection kit (Amersham Biosciences).

In Vivo Isoproteidase Assay—To confirm that Dub-1A has isopeptidase activity, the inhibitory effect of ubiquitin aldehyde on the deubiquitinizing activity of Dub-1A was analyzed. For the assay, COS-7 cells were transfected with 10 μg of pcDNA3-Myc-Dub-1A using LipofectAMINE according to the manufacturer's protocol. After 36 h, cells were washed with cold phosphate-buffered saline before application of lysis buffer (20 mM Tris (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl₂) and protease inhibitor mixture (Roche Applied Science). Lysates were then rotated at 4 °C for 1 h followed by centrifugation at 13,000 rpm for 15 min. The supernatants were immunoprecipitated with anti-Myc antibody and combined with protein A/G-Sepharose (Amersham Biosciences), followed by rotation at 4 °C for 4 h. The beads were then washed twice with lysis buffer containing 150 mM NaCl, once using lysis buffer containing 500 mM NaCl, and three times with reaction buffer (50 mM Hepes (pH 7.8), 0.5 mM EDTA, 0.01% Brij, and 3 mM dithiothreitol) before 50% of the beads were used in hydrolysis reactions. To inhibit deubiquitinating activity, Dub-1A was preincubated in the presence of 2 μM ubiquitin aldehyde (Affinity Research Products) for 30 min at 37 °C. Polyubiquitin chains (Ub5, -7, -11) were then used as substrates for hydrolysis at a concentration of 1 μM as described previously (29). Following hydrolysis, ubiquitins were detected by Western blotting using anti-ubiquitin antibody (Sigma), horseradish peroxidase-conjugated secondary antibody (Zymed Laboratories Inc.), and the ECL detection kit.

In Vivo Co-immunoprecipitation for Ubiquitination Assay—For in vivo co-immunoprecipitation assay, NIH3T3 cells were transfected with 2 μg of pcDNA3-Myc-Dub-1A and pMT123-HA-ubiquitin. After a 48-h transfection, co-immunoprecipitation was performed, and precipitates were resolved on a 10% SDS-polyacrylamide gel and subjected to Western blot analysis. Bands were visualized with chemiluminescence (ECL detection kit).

In Vivo Transfection of Ba/F3 Cells with Wild-type (WT) Jak2 or Dominant-negative (DN) Jak2—Ba/F3 cells growing in IL-3-containing medium were transfected by electroporation. A cDNA construct (pBOS-WT-Jak2 or pBOS-DN-Jak2) and pSV2neo were applied. G418-resistant Ba/F3 subclones were isolated by limiting dilution in 96-well microtiter plates. Stable expression of WT-Jak2 or DN-Jak2 was confirmed by immunoblot analysis. Each cell line was starved of IL-3 for 8 h and restimulated with 10 pg IL-3 for 2 h. The relative expression level of Dub-1A in transfected Ba/F3 cells was then determined according to the expression level of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. The band density of DNA stained with ethidium bromide on a 1% agarose gel was measured using a Gel Doc system (Bio-Rad). Densitometric intensities were normalized to those of the glyceraldehyde-3-phosphate dehydrogenase used as a control. Expression levels were tested at least three times by independent transfection.

Isolation of an Enhancer Element and Construction of Luciferase Reporter Plasmids—A 1427-bp fragment corresponding to the promoter region of the Dub-1A gene was amplified by PCR from two BAC clones (BAC2 and BAC3). The primers used for PCR were DUB1e1 (5'-CTAG-TAAGGATATAACGG-3') and T41e5 (5'-CATTCCAGTTACGCT-GTGGC-3'). The amplified PCR product was subcloned into the pCR2.1-TOPO vector, and the putative enhancer domain (112 bp) from the promoter region of the Dub-1A gene was isolated using PCR with primers DUB1e1 and DUB1e2 (5'-CACAGGCAACAGCAGCTACCTGAAT-3'). The pG2 promoter plasmid (Promega), which contains the SV40 basal promoter upstream of the luciferase reporter gene. Mutations of Ets, AP-1, and GATA in the Dub-1A enhancer region generated by PCR using mutant oligonucleotide primers are indicated in Fig. 7A.

Transient Transfection and Transactivation Experiments—Transient transfection of Ba/F3 cells and luciferase assays were performed as described previously (30), with minor modifications. Briefly, Ba/F3 cells were washed with phosphate-buffered saline free of serum and IL-3 and cultured in plain RPMI 1640 medium for 4 h. Afterward, they were resuspended at 1 × 10⁶ cells/0.8 ml of RPMI 1640 medium and transferred to an electroporation cuvette. The cells were then electroporated with 5 μg of the pMT123-HA-Dub-1A expression plasmid along with 1 μg of a cytomegalovirus promoter-driven β-galactosidase reporter construct to monitor transfection efficiencies. After electroporation with a Bio-Rad electroporator (350 V, 960 microfarads), the cells were divided into two pools and either restimulated with 10 pg IL-3 or maintained in IL-3 for 24 h.
IL-3 for 4 h or left untreated. Luciferase and β-galactosidase levels were then analyzed according to manufacturer specifications (luciferase assay kit (Analytical Luminescence Laboratory) and Galacto-Light kit (Tropix), respectively). Each luciferase reporter construct was tested at least three times by independent transfection.

**RESULTS**

Cloning and Expression of a Novel Dub-1A Gene, the Smallest Dub Subfamily Member—We have previously screened a murine genomic BAC library using Dub-2-specific primers and isolated three BAC clones that may contain a putative genomic Dub-2 gene (25). Unexpectedly, one of these BAC clones (BAC1) contains the Dub-2A gene, which is highly homologous to Dub-2 (25), and the other two BAC clones (BAC2 and BAC3) were subjected to further analysis in this study. Digestion of both BAC clones with different restriction enzymes revealed that they contain the same genomic fragment (data not shown).

Sequence analysis of amplified PCR products derived from these clones showed a novel Dub gene (Dub-1A) composed of two exons and one intron (Fig. 1A). This is similar to the structural organization of the genomic DNAs for other Dub subfamily members, including Dub-1, Dub-2, and Dub-2A (2, 24, 25).

Because the amino acid sequences of Dub subfamily members are highly homologous to one another, we next compared the predicted amino acid sequence of DUB-1A with those previously reported for DUB-1, DUB-2, and DUB-2A (Fig. 2). DUB-1A has 84.5% amino acid identity to DUB-1, 84.7% amino acid identity to DUB-2, and 85.3% amino acid identity to DUB-2A. Like other known ubiquitin-processing protease enzymes,

**Fig. 1. A, schematic representation of the Dub-1A genomic structure. The indicated primers were used for genomic PCR of the 5′-region, open reading frame region, and 3′-region using murine BAC clones as templates. B, nucleotide and predicted amino acid sequences of the Dub-1A gene.**

Like other Dub subfamily members (Dub-1, Dub-2, and Dub-2A), the Dub-1A gene contains two exons. A putative TATA box (position −110) is indicated. The enhancer domain extending from positions −1511 to −1400 contains one ETS protein-binding site, and one GATA site (underlined). The sequence of the Dub-1A gene has been submitted to the GenBank™/EBI Data Bank (accession number AJ257250).

Cytokine-inducible DUB-1A Is Polyubiquitinated

![Diagram](image-url)
including DUB subfamily members, DUB-1A contains three conserved domains, which are cysteine, histidine, and aspartic acid domains (Fig. 2). Previous reports revealed that these domains are required for deubiquitinating enzyme activity, indicating that they are involved in constructing the active sites of the enzymes (25, 31). Interestingly, both DUB-1A and DUB-1 lack a short hypervariable region present in DUB-2 and DUB-2A (amino acids 444–463, KHR(I/N)NEILPQEQNHQK(A/T)GQS) (Fig. 2). The cellular function of this region in DUB-2 and DUB-2A has not yet been elucidated. As shown in Fig. 2, the C terminus of DUB-1A is much shorter than those of other DUB subfamily members. These data support the observation that deubiquitinating enzymes function as long as the polypeptide contains those three conserved amino acids (Cys, His, and Asp).

Expression of Dub-1A in Various Tissues and Cytokine-dependent Expression in B- and T-lymphocytes—Due to the presence of Dub-1 in B-lymphocytes, we analyzed the expression pattern of Dub-1A mRNA by RT-PCR using Dub-1A-specific primers (Fig. 1A). We designed specific primers for Dub-1 (1A-S1 and 1A-S2) and demonstrated that they specifically amplified Dub-1A, whereas the degenerate PCR primers recognized all DUB subfamily members (Fig. 3A). Therefore, we used these primers for RT-PCR to determine whether Dub-1A mRNA is expressed in various murine tissues. The sequences of the PCR products showed that Dub-1A is expressed in brain, heart, lung, kidney, ovary, spleen, and B-lymphocytes (Fig. 3B). Interestingly, the expression of Dub-1A in B-lymphocytes was IL-3-dependent. Therefore, we next analyzed the minimal concentration of IL-3 required for Dub-1A to be expressed. After the depletion of IL-3 for 8 h, cells were cultured with different concentrations of IL-3. Dub-1A was not expressed until 10 pM IL-3 or higher was added to the culture medium (Fig. 3C). Dub-1A was constitutively expressed in CTLL-2 cells, indicating that the IL-2 cytokine is not required for the expression of Dub-1A in CTLL-2 cells (Fig. 3D).

Because Dub subfamily members are immediate-early genes, we tested whether Dub-1A is expressed very rapidly in response to cytokine stimulation. Fig. 3D shows that Dub-1A was expressed within 30 min and was maximally expressed in 2 h. This is similar to the expression pattern of other Dub subfamily members, including Dub-1 and Dub-2. After 2 h, the expression of Dub-1A slowly decreased and disappeared after 10 h.

Dub-1A Encodes a Functional Deubiquitinating Enzyme—To determine whether Dub-1A has deubiquitinating enzyme activity, we examined the ability of DUB-1A to cleave ubiquitin from polyubiquitin chains conjugated with proteins via isopeptide bonds under in vivo conditions. HA-tagged ubiquitin was transiently expressed in NIH3T3 fibroblast cells with and without Myc-tagged Dub-1A and its mutant form (Dub-1A(C60S)), followed by immunoblot analysis of the cell extracts using anti-HA antibody (Fig. 4A). The expression of Dub-1A almost completely deubiquitinated polyubiquitinated protein targets in the cells (Fig. 4A, lane 5). However, de-
ubiquitination was hardly seen in cells expressing *Dub-1A* (C60S) (lane 6), in which the cysteine was replaced in the catalytic triad with serine.

In addition, we expressed DUB-1A as a GST fusion protein to determine whether DUB-1A has deubiquitinating enzyme activity. Immunoblot assays showed that the cDNA clone encoding the GST-DUB-1A fusion protein resulted in cleavage of Ub-Methionine-β-galactosidase (Fig. 4B, lane 2) to an extent comparable with that observed with GST-DUB-1 (lane 4), GST-DUB-2 (lane 6), and GST-DUB-2A (lane 8). As a control, cells transformed with the pGEX vector failed to cleave Ub-Methionine-β-galactosidase (lane 1). A mutant form of the DUB-1A polypeptide containing a C60S mutation was unable to cleave the Ub-Methionine-β-galactosidase substrate (lane 3). These results demonstrate that DUB-1A has deubiquitinating enzyme activity and that cysteine at position 60 is essential for its thiol protease activity, as shown for other DUB subfamily members (2, 24, 25). These results in *vivo* and *in vitro* indicate that DUB-1A has isopeptidase activity.

To confirm that DUB-1A has isopeptidase activity, the effect of ubiquitin aldehyde, a specific inhibitor of deubiquitinating enzymes, on the deubiquitinating activity of DUB-1A was investigated. In the presence of ubiquitin aldehyde, DUB-1A proteins expressed in COS-7 cells were not capable of hydrolyzing the ubiquitin from branched polyubiquitin chains (Fig. 4C), suggesting that DUB-1A has isopeptidase activity.

**Ubiquitination of the DUB-1A Protein**—Because it has been reported that several proteins, including IκB, undergo phosphorylation by kinase before ubiquitination, we therefore analyzed whether DUB-1A is phosphorylated and ubiquitinated or not. Immunoprecipitation assay revealed that the DUB-1A protein was not tyrosine-phosphorylated (data not shown). However, Myc-tagged DUB-1A was coprecipitated with HA-tagged ubiquitin in NIH3T3 cells (Fig. 5, A and B), suggesting that DUB-1A itself is ubiquitinated. It remains to be determined whether the phosphorylation takes place before ubiquitination at either serine or threonine or both amino acids.

**JAK2 Signaling Is Required for Induction of DUB-1A Expression**—To investigate DUB-1A induction in Ba/F3 cells, we investigated the requirement of JAK2 kinase signaling because DUB-1A was expressed in response to IL-3 stimulation in B-lymphocytes (Fig. 3, B and C). Transfection with WT-Jak2 revealed higher expression of DUB-1A (Fig. 6A, lane 2), whereas DUB-1A expression was reduced in Ba/F3 cells transfected with DN-Jak2 lacking the C-terminal tyrosine kinase domain (lane 3), suggesting that DUB-1A functions downstream of JAK2 kinase and requires the presence of WT-JAK2 kinase. This experiment was performed at least three times by independent transfection. This is similar to the expression of Dub-1, which is dependent on JAK2 (30). As shown in Fig. 6B, the expression of DUB-1A in Ba/F3 cells transfected with DN-JAK2 was ~30% less compared with that in Ba/F3 cells without transfection.

**The DUB-1A Gene Contains a Cytokine-inducible Enhancer Element**—We have previously reported that both the Dub-1 and Dub-2A genes contain a cytokine-responsive enhancer element (2, 25). It has been demonstrated that the minimal enhancer elements of Dub-1 and Dub-2A are 112 and 100 bp, respectively (25). In an attempt to identify an enhancer region in the Dub-1A gene, we compared the 5′-sequences of Dub-1, Dub-2A, and Dub-1A within the enhancer element (Fig. 7A). Interestingly, they are highly homologous to each other and contain Ets, AP-1, and GATA sequences, suggesting the conserved function of the enhancer.

Transcriptional reporter assays in the murine hematopoietic pro-B-lymphocyte cell line (Ba/F3 cells) were performed to test the putative enhancer activity of the Dub-1A region (Fig. 7B). Ba/F3 cells are dependent on murine IL-3 for growth and survival. Ba/F3 cells were transfected with various reporter constructs, including mutant Dub-1A enhancer domains for
Ets, AP-1, and GATA sequences (Fig. 7A), and IL-3-induced activities were measured (Fig. 7B). As shown in Fig. 7B, the enhancer sequence for Dub-1A had enhancer activity, and mutations at two AP-1 sites and the Ets site in the Dub-1A sequence did not show the activity. This is similar to previous reports that revealed the requirement for AP-1 and Ets sequences in Dub-1 and Dub-2 (25, 32). Therefore, it is possible that both Ets and AP-1 (but not GATA) sequences are required for the induction of enhancer activity in Dub subfamily members.

**DISCUSSION**

It is becoming clear that the regulation of ubiquitination and deubiquitination for protein degradation is essential for cellular processes, including cell proliferation and differentiation (3, 20). A number of diseases have been found to be associated with aberrant protein degradation. These include Parkinson’s disease, Alzheimer’s disease, Angelman syndrome, Huntington’s disease, and cystic fibrosis (33), suggesting important roles of protein degradation in normal cellular processes. We have previously reported a hematopoiesis-specific cytokine-inducible gene encoding a growth regulatory deubiquitinating enzyme (DUB-1) (2). Based on the fact that the overexpression of DUB-1 results in cell cycle arrest prior to S phase (2), it is possible that the aberration of Dub expression may lead to abnormal proliferation of lymphocytes, causing leukemia in mammals. Therefore, detailed analysis of deubiquitinating enzymes involved in the regulation of protein degradation is required to better understand their cellular functions.

Recently, three Dub genes have been described and categorized into a novel class of deubiquitinating enzymes: Dub-1 (2), Dub-2 (24), and Dub-2A (25). Both Dub-1 and Dub-2 were identified as hematopoiesis-specific immediate-early genes that are rapidly induced in response to cytokines (2, 24). Dub-1 is expressed in B-lymphocytes by stimulation with IL-3, IL-5, and granulocyte/macrophage colony-stimulating factor (2). Dub-2 is expressed in T-lymphocytes by stimulation with IL-2 (24). However, Dub-2A is expressed not only in T-lymphocytes, but also in embryonic stem cells in mouse (25). Interestingly, all DUB enzymes revealed deubiquitinating activity and contain three conserved domains (Cys, His, and Asp) throughout the N terminus. In the absence of these domains, the enzymes are not capable of cleaving ubiquitin, suggesting a critical role in catalytic enzyme reaction. Recently, the structure of the catalytic domain of herpesvirus-associated ubiquitin-specific protease, which deubiquitinates and stabilizes the tumor suppressor p53, has been investigated (34). This investigation revealed that the catalytic core domain of the herpesvirus-associated ubiquitin-specific protease enzyme binds ubiquitin al-
dehyde, leading to a dramatic conformational change in the active site similar to the one for herpesvirus-associated ubiquitin-specific protease binding to its substrate (34). Interestingly, the conserved aspartic acid residue participating in the catalytic triad was found downstream of the conserved histidine residues, indicating that the conserved aspartic acid at position 133, localized upstream of the conserved histidine residues in DUB-1 and DUB-2, is involved in the formation of the oxyanion hole instead of catalytic activity. The detailed molecular mechanisms for these enzymes should be investigated to develop pharmaceutical reagents that target these mechanisms in signal transduction pathways involved in a number of cellular processes, including cell proliferation and differentiation.

In this study, we have identified a novel Dub-1A gene from BAC clones that encodes another member of the DUB subfamily of deubiquitinating enzymes. Dub-1A is composed of two exons and one intron and encodes an enzyme highly homologous to other DUB subfamily members (Table I). It has been reported that Dub genes are located in the region of murine chromosome 7 with a tandem repeat array (24). Because both complete Dub-1A and Dub-1 genes are found in the same BAC clones, we expect that Dub-1A is also located in chromosome 7. The sequence similarity among Dub subfamily members and chromosomal co-localization suggest that the Dub genes arose by a tandem duplication of an ancestral Dub gene. Interestingly, Dub-1A is expressed in various tissues, including B- and T-lymphocytes, and encodes the shortest form of DUB protein among subfamily members of deubiquitinating enzymes. DUB-1A, like DUB-1, does not contain the hypervariable domain that is present in both DUB-2 and DUB-2A (Fig. 2). Because the C terminus of Dub-1A tends to encode different amino acids compared with other DUB subfamily members, we propose that the C terminus rather than the N terminus has substrate specificity. This supports the previous observation that deubiquitinating enzymes in signal transduction pathways involved in a number of cellular processes, including cell proliferation and differentiation.
activity is retained upon deletion of the C terminus as long as the three conserved domains are intact (25, 31).

It has been reported that JAK2 kinase and the Ras-Raf-MEK-ERK kinase signaling pathway are required for induction of the murine Dub-1 gene, even though the presence of another signaling pathway may be involved (30). Interestingly, the minimal interleukin-3-responsive element of the Dub-1 gene contains cytokine-inducible enhancer activity, but lacks a consensus sequence for STAT binding. This indicates that Dub-1 is expressed in a JAK2-dependent, but STAT5-independent pathway (30). In the case of the Dub-1A enhancer sequence, the consensus sequence for STAT binding is also not present. However, blocking JAK2 signaling inhibits the expression of Dub-1A (Fig. 6, A and B), indicating the requirement of JAK2 signaling for Dub-1A to be expressed. Because many receptor tyrosine kinases are regulated not only by phosphorylation and dephosphorylation, but also by ubiquitination and deubiquitination, it is possible that signaling pathways mediated by these receptors can be modulated by the ubiquitination status of them. Therefore, finding the molecular mechanisms for the regulation of protein degradation via ubiquitination and deubiquitination in receptor tyrosine kinase-mediated signal transduction pathways will contribute to understanding the regulation of cell proliferation and differentiation. Interestingly, Dub-1A itself is ubiquitinated (Fig. 5, A and B), suggesting that this protein is also regulated by proteasome-mediated degradation when it is no longer necessary within cells.

Because Dub-1 and Dub-2A contain a cytokine-inducible enhancer element, we have further characterized the enhancer element of Dub-1A. Transcriptional reporter assays in Ba/F3 cells revealed that two AP-1 sites and one Ets site are required for Dub-1A enhancer activity. This is similar to Dub-1 and Dub-2A (25, 31), suggesting that they play a key role in responding to cytokine stimuli. Even though substrates for each DUB enzyme remain to be found, it will be helpful to identify ways of regulating their expression. This will give us insights into the regulation of lymphocyte proliferation and immune responses in vivo. It has been suggested that Dub-2 induced by IL-2 might be involved in the regulation of T cell receptor clustering for the supramolecular activation complex by deubiquitinating p85, the phosphatidylinositol 3-kinase adaptor subunit (27). The functional roles of DUB enzymes in signal transduction in immunity have to be investigated to provide new possibilities for designing therapeutic drugs.

**Acknowledgments**—We thank Alan D. D’Andrea (Harvard Medical School) and members of the Cell and Gene Therapy Research Institute of the Pochon CHA University for critical comments on the manuscript.

---

**TABLE I**

Percent amino acid identity among murine DUB subfamily members

|          | DUB-1A | DUB-1 | DUB-2 | DUB-2A |
|----------|--------|-------|-------|--------|
| %        |        | %     | %     | %      |
| DUB-1A   | 100    | 84.5  | 84.7  | 85.3   |
| DUB-1    | 100    | 88.4  | 87.1  |        |
| DUB-2    | 100    | 95.8  |       |        |
| DUB-2A   | 100    |       |       |        |

**Fig. 7. Identification and functional analysis of the cytokine-inducible enhancer of the Dub-1A gene.** A, shown is a comparison of the minimal enhancer regions of Dub-1A, Dub-1, and Dub-2A. The enhancer region of Dub-1A contains one Ets site, two AP-1 sites, one TG protein-binding site, and one 5'-GATA site. Mutation sites in motifs are indicated with arrows. B, luciferase activity was analyzed in Ba/F3 cells transfected with the indicated constructs. The cells were starved and restimulated without (open bars) or with (closed bars) 10 pm IL-3. Luciferase assays were performed after 8 h of induction. luc/β-gal, luciferase/β-galactosidase; mut, mutant.
REFERENCES

1. Moazed, D., and Johnson, A. D. (1996) Cell 86, 667–677
2. Zhu, Y., Carrell, M., Papa, F. R., Hochstrasser, M., and D’Andrea, A. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3275–3279
3. Ciechanover, A. (1996) EMBO J. 17, 7151–7160
4. Cadavid, A. L., Ginzel, A., and Fischer, J. A. (2000) Development 127, 1727–1736
5. Pantaleon, M., Kanai-Azuma, M., Mattick, J. S., Kaiuchi, K., Kaye, P. L., and Wood, S. A. (2001) Mech. Dev. 109, 151–160
6. Park, K. C., Kim, J. H., Choi, E. J., Min, S. W., Rhee, S., Baek, S. H., Chung, S. S., Bang, O., Park, D., Chiba, T., Tanaka, K., and Chung, C. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9733–9738
7. Gewies, A., and Grimm, S. (2003) Cancer Res. 63, 682–688
8. Hochstrasser, M. (1996) Cell 84, 813–815
9. Baumeister, W., Walz, J., Zühl, F., and Seemüller, E. (1998) Cell 92, 367–380
10. Wilkinson, K. D. (2000) Semin. Cell Dev. Biol. 11, 141–148
11. Varshavsky, A. (1997) Trends Biochem. Sci. 22, 383–387
12. Weiseman, A. M. (2001) Nature 2, 169–178
13. Ciechanover, A., Oriz, A., and Schwartz, A. L. (2000) BioEssays 22, 442–451
14. Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (1999) Cell 96, 635–644
15. Coux, O., Tanaka, K., and Gelberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847
16. Hochstrasser, M. (1996) Annu. Rev. Genet. 30, 405–439
17. Wilkinson, K. D. (1997) FASEB J. 11, 1245–1256
18. Holz, H., Kapelari, B., Kellermann, J., Seemüller, E., Sumegi, M., Udvardy, A., Medalia, O., Sperling, J., Muller, S. A., Engel, A., and Baumeister, W. (2000) J. Cell Biol. 150, 119–130
19. Leggett, D. S., Hanna, J., Borodovsky, A., Crossas, B., Schmidt, M., Baker, R. T., Walz, T., Ploegh, H., and Finley, D. (2002) Mol. Cell 10, 495–507
20. D’Andrea, A. D., and Pellman, D. (1998) Crit. Rev. Biochem. Mol. Biol. 33, 337–352
21. Chung, C. H., and Baek, S. H. (1999) Biochem. Biophys. Res. Commun. 266, 633–640
22. Borodovsky, A., Ova, H., Kolli, N., Gan-Erdene, T., Wilkinson, K. D., Ploegh, H. L., and Kessler, B. M. (2002) Chem. Biol. 9, 1149–1159
23. Baek, K. H. (2000) Exp. Mol. Med. 35, 1–7
24. Zhu, Y., Lambert, K., Corless, C., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and D’Andrea, A. D. (1997) J. Biol. Chem. 272, 51–57
25. Baek, K.-H., Mondoux, M. A., Jaster, R., Fire-Levin, E., and D’Andrea, A. D. (2001) Blood 98, 636–642
26. Migone, T. S., Humbert, M., Rascle, A., Sanden, D., D’Andrea, A., and Johnston, J. A. (2001) Blood 98, 1935–1941
27. Ben-Neriah Y. (2002) Nat. Immunol. 3, 20–26
28. Papa, F. R., and Hochstrasser, M. (1993) Nature 366, 313–319
29. Evans, P. C., Smith, T. S., Lai, M.-J., Williams, M. G., Burke, D. F., Heyninck, K., Kreike, M. M., Seyaart, R., Blundell, T. L., and Kilshaw, P. J. (2003) J. Biol. Chem. 278, 21889–21896
30. Jaster, R., Zhu, Y., Pless, M., Bhattacharya, S., Mathey-Prevot, B., and D’Andrea, A. D. (1997) Mol. Cell. Biol. 17, 3364–3372
31. Lee, J.-H., Kim, Y.-S., Kim, M., and Baek, K.-H. (2001) Am. J. Hematol. 67, 270–272
32. Jaster, R., Baek, K.-H., and D’Andrea, A. D. (1999) Biochim. Biophys. Acta 1446, 308–316
33. Layfield, R., Alban, A., Mayer, R. J., and Lewe, J. (2001) Neuropathol. Appl. Neurobiol. 27, 171–179
34. Hu, M., Li, P., Li, M., Li, W., Yao, T., Wu, J. W., Gu, W., Cohen, R. E., and Shi, Y. (2002) Cell 111, 1041–1054