DUB-2A, a new member of the DUB subfamily of hematopoietic deubiquitinating enzymes

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Protein ubiquitination is an important regulator of cytokine-activated signal transduction pathways and hematopoietic cell growth. Protein ubiquitination is controlled by the coordinate action of ubiquitin-conjugating enzymes and deubiquitinating enzymes. Recently a novel family of genes encoding growth-regulatory deubiquitinating enzymes (DUB-1 and DUB-2) has been identified. *DUB*s are immediate-early genes and are induced rapidly and transiently in response to cytokine stimuli. By means of polymerase chain reaction amplification with degenerate primers for the *DUB-2* complementary DNA, 3 murine bacterial artificial chromosome (BAC) clones that contain *DUB* gene sequences were isolated. One BAC contained a novel *DUB* gene *(DUB-2A)* with extensive homology to *DUB-2.* Like *DUB-1* and *DUB-2,* the *DUB-2A* gene consists of 2 exons. The predicted DUB-2A protein is highly related to other DUBs throughout the primary amino acid sequence, with a hypervariable region at its C-terminus. In vitro, *DUB-2A* had functional deubiquitinating activity; mutation of its conserved amino acid residues abolished this activity. The 5′ flanking sequence of the *DUB-2A* gene has a hematopoietic-specific functional enhancer sequence. It is proposed that there are at least 3 members of the *DUB* subfamily *(DUB-1, DUB-2,* and *DUB-2A)* and that different hematopoietic cytokines induce specific *DUB* genes, thereby initiating a cytokine-specific growth response. (Blood. 2001;98: 636-642)

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Introduction

Protein ubiquitination controls many intracellular processes, including cell cycle progression.[1,2] transcriptional activation,[3] and signal transduction[4] (reviewed in Ciechanover[5] and D'Andrea and Pellman[6]). Like protein phosphorylation, protein ubiquitination is dynamic, involving enzymes that add ubiquitin (ubiquitin-conjugating enzymes) and enzymes that remove ubiquitin (deubiquitinating enzymes). Considerable progress has been made in understanding ubiquitin conjugation and its role in regulating protein degradation. Recent studies have demonstrated that regulation also occurs at the level of deubiquitination. Deubiquitinating enzymes are cysteine proteases that specifically cleave ubiquitin from ubiquitin-conjugated protein substrates. Deubiquitinating enzymes have significant sequence diversity and may therefore have a broad range of substrate specificity.

There are 2 major families of deubiquitinating enzymes, the ubiquitin-processing proteases (ubp) family[7-9] and the ubiquitin carboxy-terminal hydrolase (uch) family.[10,11] The ubp family and the uch family have also been referred to as the type 1 uch and type 2 uch families.[12] Both ubps and uchs are cysteine proteases containing an active site cysteine, aspartate, and histidine residue. Ubps vary greatly in size and structural complexity, but all contain 6 characteristic conserved homology domains.[13] Uchs, in contrast, include a group of small, closely related proteases that lack the 6 characteristic homology domains of the ubps.[11]

Little is known regarding the precise cellular function of ubps and uchs. For instance, despite the broad range and structural diversity of these enzymes, only a few specific candidate substrates have been identified.[14-16] Also, whether these enzymes act exclusively on ubiquitinated substrates or on substrates with ubiquitin-like modifications, such as SUMO-1[17,18] and NEDD8,[19] remains unknown. Recently, a distinct family of cysteine proteases, acting on SUMO-1–conjugated substrates, has been identified.[20] Finally, the precise cellular level of action of these enzymes is unknown. Some deubiquitinating enzymes may act before the proteasome, thereby removing ubiquitin and rescuing a substrate protein from degradation.[6] Other deubiquitinating enzymes may act as a component of the proteasome, thereby promoting the net degradation of a specific ubiquitinated substrate.[21]

Despite this lack of information regarding substrate specificity, substrate selection, and level of action, it is clear that some deubiquitinating enzymes exert distinct growth-regulatory activities or growth effects on cellular differentiation. The tre-2 oncoprotein, for example, is a deubiquitinating enzyme with transforming activity.[8,22] The FAF protein is a ubp that regulates *Drosophila* eye development.[23] Other ubps, such as UBP3[24] and *Drosophila* ubp-64E,[25] play an important role in transcriptional silencing.

We have recently identified a hematopoietic-specific growth-regulatory subfamily of ubps, referred to as *DUB*s.[26,27] *DUB-1* was originally cloned as an immediate-early gene induced by the cytokine interleukin-3 (IL-3). Several lines of evidence suggest that *DUB-1* plays a growth-regulatory role in the cell. First, the expression of *DUB-1* has the characteristics of an immediate-early gene. Following IL-3 stimulation, the *DUB-1* messenger RNA (mRNA) is rapidly induced and is superinduced in the presence of

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cyclohexamide. Second, high-level expression of *DUB-1* from an inducible promoter results in cell cycle arrest prior to S phase. This result suggests that *DUB-1* controls the ubiquitin-dependent proteolysis or the ubiquitination state of an important regulator at the G_1/S transition of the cell cycle. Finally, the induction of DUB proteins may be a general feature of the response to cytokines. Another family member, *DUB-2,* is induced by IL-2.[28]

DUB proteins are highly related to each other, not only within the 6 characteristic ubp domains, but also throughout their protein sequence. One short peptide region within the C-terminal extension of *DUB* family members shows remarkable sequence diversity. This "hypervariable region" may have a role in the recognition of specific substrates.[28] A tandem repeat of *DUB* genes maps to a region of murine chromosome 7,[28] suggesting that the *DUB* subfamily arose by tandem duplication of an ancestral *DUB* gene.

In the current study, we have cloned the complementary DNA (cDNA) and gene for a new member of the hematopoietic-specific *DUB* subfamily of deubiquitinating enzymes. Because the new *DUB* is highly related to *DUB*-2, in terms of its sequence, genomic organization, and expression pattern, we have named the new gene *DUB-2A. DUB-2A* encodes a functional deubiquitinating enzyme, which is rapidly induced in response to cytokine stimulation of hematopoietic cells. A minimal catalytic domain in the N-terminal region of *DUB-2A* is necessary and sufficient for functional activity, and the carboxy-terminal region, which is hypervariable among *DUB*s, is not required for activity. Moreover, we propose that *DUB-2A* regulates cellular growth by controlling the ubiquitin-dependent degradation or the ubiquitination state of an unknown intracellular growth-regulatory protein.

Materials and methods

Cells and cell culture

Ba/F3 is an IL-3–dependent murine pro-B cell line.[26] Ba/F3 cells were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum and 10 pM of murine IL-3. Growth conditions for murine E9 cells, 32D cells, and embryonal stem (ES) cells have been previously described.[26]

Southern and Northern blot analyses

For Southern blots, genomic DNA (10 to 15 μg) was digested with the *Eco*RV, electrophoresed on 1% agarose gel, and blotted onto Duralon-UV membranes (Stratagene, La Jolla, CA). The 0.8-kilobase (kb) probe, generated from the digestion of the 15-kb genomic *DUB-2* DNA with Kpn I and Hind III, purified from agarose gels (Qiagen, Chatsworth, CA), was radiolabeled and hybridized for 2 hours to the membrane in a 65°C oven. Hybridized filters were washed at 65°C in 1 × sodium chloride/sodium acetate hybridization solution (SSC) and 0.2% sodium dodecyl sulfate (SDS) for 15 minutes and washed again in 0.5 × SSC and 0.2% SDS for 15 minutes. For Northern blots, RNA samples (10 to 20 μg) were electrophoresed on denaturing formaldehyde gels and blotted onto Duralon-UV membranes. The 0.8-kb probe derived from 3′-UTR was radiolabeled and hybridized to the membrane as Southern blotting. Reverse transcription–polymerase chain reaction (RT-PCR) for analysis of *DUB-2A* mRNA expression was performed as previously described.[26]

Isolation of the genomic *DUB-2A* gene and construction of luciferase reporter plasmids

The murine genomic library in pBeloBAC11 vector was screened by genomic PCR by means of 2 primers (Bam5′, GCGGATCCTTTGAAGAGGTCTTTGGAAA, and Xho3′, ATCTCGAGGTGTCCACAGGAGCCTGTGT) derived from the *DUB-2* DNA sequence. By means of the same primers, genomic PCR products from 1 of the 3 positive bacterial artificial chromosome (BAC) clones were generated and sequenced.

DUB-1 and *DUB-2A* enhancer elements were identified and subcloned into the PGL2Promoter plasmid (Promega, Madison, WI), which contains the simian virus SV40 basal promoter upstream of the luciferase reporter gene. The enhancer region of the *DUB-2A* gene corresponds to the minimal IL-3 response element of the murine *DUB-1* gene (nucleotides −1528 to −1416), which has previously been described.[29,30]

Deubiquitination assay

A deubiquitination assay, based on the cleavage of ubiquitin–β-galactosidase (ub-β-gal) fusion proteins, has been described previously.[8] A 1638–base pair (bp) fragment from the wild-type *DUB-2A* cDNA (corresponding to amino acids 1 to 545) and a cDNA containing a missense mutant form, *DUB-2A* (C60S [single-letter amino acid codes]), were generated by PCR and inserted, in frame, into pGEX-2TK (Pharmacia, Piscataway, NY), downstream of the glutathione *S*-transferase (GST) coding element. Ub-Met-β-gal was expressed from a pACYC184-based plasmid. Plasmids were cotransfomed into MC1061 *Escherichia coli*. Plasmid-bearing *E coli* MC1061 cells were lysed and analyzed by immunoblotting with a rabbit anti–β-gal antiserum (Cappel, Durham, NC), a rabbit anti-GST antiserum (Santa Cruz Biotechnology, CA), and the ECL system (Amersham, Buckinghamshire, United Kingdom).

Transient transfection and transactivation experiments

All plasmid DNAs were purified with Qiagen columns. Transient transfections of Ba/F3 cells and luciferase reporter gene assays were performed as previously described,[27] with the following modification. Ba/F3 cells were washed free of serum and IL-3 and cultured in plain RPMI for 2 hours. Afterwards, they were resuspended at 1×10^7 cells per 0.8 mL RPMI and transferred to an electroporation cuvette. Cells were incubated with 10 μg of the indicated luciferase reporter vector, along with 1 μg of a cytomegalovirus-promoter-driven β-galactosidase (β-gal) reporter gene construct to monitor transfection efficiencies. After electroporation with a Bio-Rad (Hercules, CA) electroporator (350 V, 960 microfarads [μF]), cells were divided into 2 pools and either restimulated with 10 pM IL-3 for 4 hours or left untreated. Then, luciferase and β-galactosidase levels were assayed by, respectively, the Luciferase assay kit (Analytical Luminescence Laboratory, San Diego, CA) and the Galacto-Light Kit (Tropix, Bedford, MA) according to vendor specifications. Each luciferase reporter construct was tested at least 3 times by independent transfection.

Isolation of an enhancer sequence from the BAC clone containing the *DUB-2A* gene

A 1516–base pair fragment, corresponding to the promoter region of the *DUB-2A* gene, was amplified by PCR from the *DUB-2A* BAC clone. Primers used were *DUB*1e1 (5′-CTAGTAAGGATATAACAGG-3′) and T14/CS (5′-CATTCAGGTAGCAGCTGTTGCC-3′). The amplified PCR product was subcloned into a pCR2.1-TOPO vector and sequenced. The 100-bp fragment derived from the promoter region was further subcloned into the pGL2Promoter.

Results

Identification and cloning of a novel *DUB* gene, *DUB-2A*

We have previously characterized a genomic clone of the murine *DUB-2* gene[28] (Figure 1A). In an attempt to isolate additional *DUB-2* genomic clones with longer 5′ and 3′ genomic sequence, we screened a murine genomic BAC library, using PCR with *DUB-2*–specific primers (Figure 1A). We isolated 3 BAC clones that yielded a 1.5-kb PCR product, consistent with the presence of a *DUB* sequence. Two of the BAC clones (BAC2 and BAC3) contained the *DUB-2* gene. An additional BAC clone (BAC1)

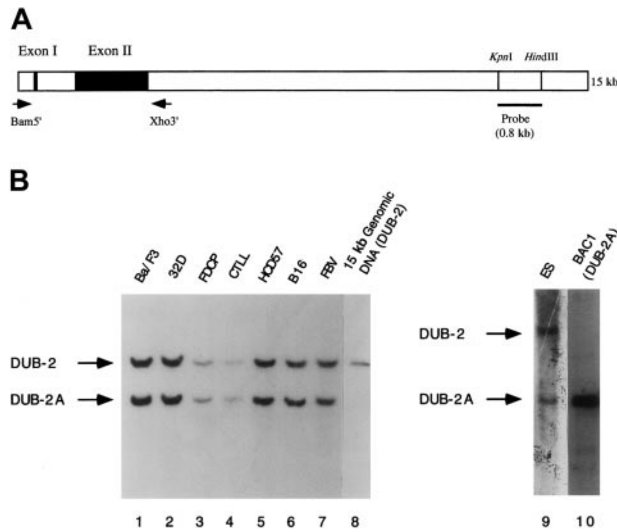

Figure 1. Identification of the murine *DUB-2* and *DUB-2A* genes by Southern blot analysis. (A) Schematic representation of the *DUB-2* gene. The indicated primers were used to amplify the *DUB-2* and *DUB-2A* genes from murine BAC clones by PCR. The indicated 800-bp probe was used for Southern blot analysis in panel B. (B) Genomic DNA from the indicated murine cell lines or BAC clones was restriction-digested with *Eco*RV, electrophoresed, blotted to nitrocellulose, and probed with the ^{32}P-labeled probe. Lanes 1 to 7 contain *Eco*RV-digested genomic DNA from the indicated murine cell lines. Ba/F3 and 32D cells were derived from Balb/c mice. Cytotoxic T-lymphocyte line (CTLL) cells were derived from C57BL mice. HCD57 cells were derived from National Institutes of Health (NIH) Swiss mice. B16 cells were derived from B16 mice. FVB cells were derived from FVB mice. ES cells were derived from 129SV mice. Lanes 8 and 10 contain digested DNA from either a *DUB-2* genomic clone or a *DUB-2A* (BAC1) genomic clone, respectively. The band corresponding to the *DUB-2* and *DUB-2A* genes is indicated.

contained a different *DUB* gene (*DUB-2A*), which was subjected to further analysis.

To distinguish the new *DUB* gene from *DUB-2*, the BAC1 clone was analyzed by Southern blot analysis (Figure 1B). In parallel, we analyzed restriction enzyme–digested genomic DNA samples from multiple murine cell lines, corresponding to 6 independent strains of mice (lanes 1 through 7). We also analyzed genomic DNA from a *DUB-2* genomic clone (lane 8). For analysis, we used a ^{32}P-labeled DNA probe from the indicated region downstream of the *DUB-2* gene (Figure 1A). This labeled probe identified 2 distinct bands in genomic DNA from all mouse species tested (Figure 1B, lanes 1 through 7). The genomic *DUB-2* clone yielded the upper band (lane 8), but the genomic *DUB-2A* clone (BAC1) yielded the lower band (lane 10). Taken together, these results demonstrate that *DUB-2* and *DUB-2A* are distinct genes found in the genome of multiple mouse strains.

The structure of the genomic *DUB-2A* gene was further examined by PCR of various regions of the BAC1 clone (Figure 2B). Distinct regions of the *DUB-2A* gene, including the 5′ and 3′ genomic sequences, were amplified by PCR with the indicated oligonucleotide primer pairs (Figure 2A). Sequencing of these amplified PCR products indicated that the *DUB-2A* gene is composed of 2 exons and 1 intron (Figure 2B) and therefore has a structural organization identical to that of the *DUB-2* gene.[28] The *DUB-2A* gene is predicted to encode a protein of 545 amino acids. Exon 1 encodes the 9 amino acid amino terminal region of *DUB-2A,* and exon 2 encodes amino acids 10 through 545. The single intron is 792 bp. The sequence of the intron-exon junction

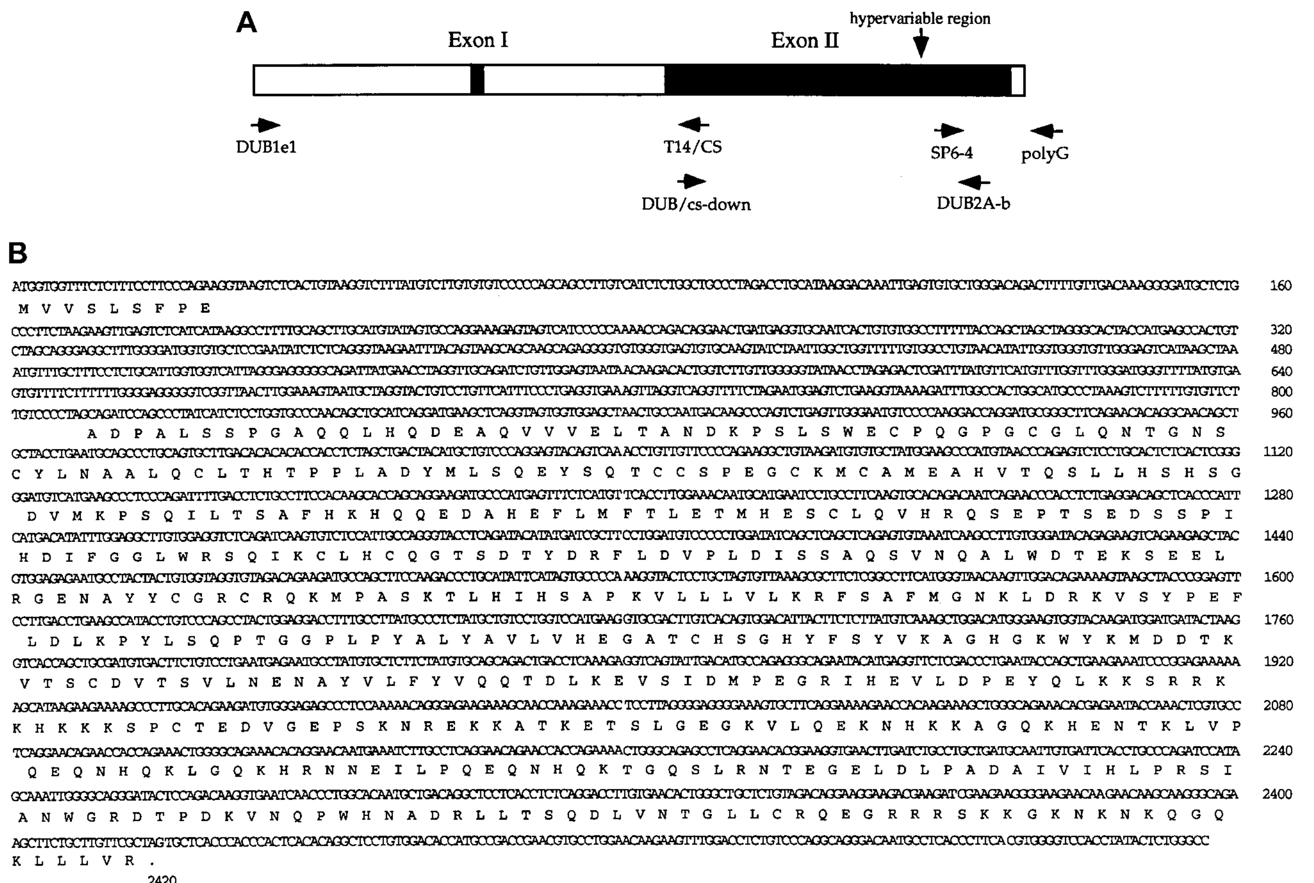

Figure 2. Characterization of the *DUB-2A* gene. (A) Schematic representation of the *DUB-2A* gene. Primer pairs used for genomic PCR of the 5′ region, open reading frame (ORF) region, and 3′ region are indicated. (B) Nucleotide and predicted amino acid sequence for the *DUB-2A* gene. The gene contains 2 exons, similar to the genomic structure of *DUB-1* and *DUB-2*.[25,26] The sequences of the *DUB-2A* gene and cDNA have been submitted to GenBank (accession number 407172).

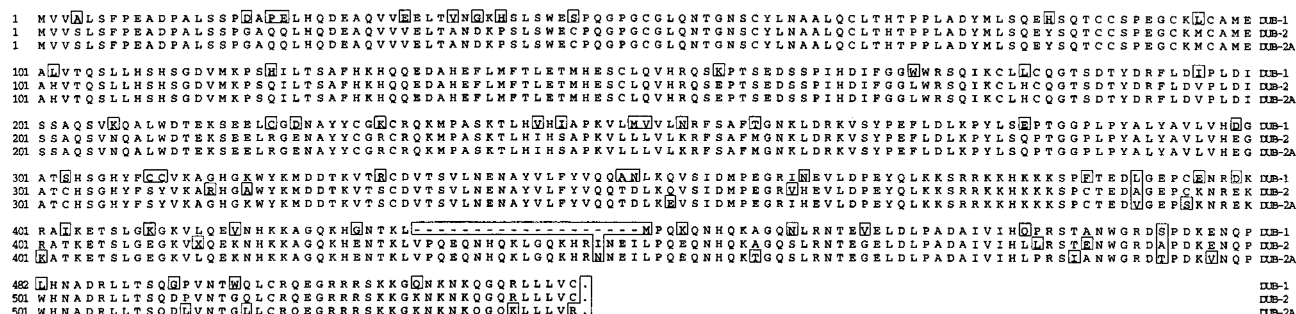

Figure 3. Amino acid alignment of DUB-1, DUB-2, and DUB-2A proteins. The predicted amino acid sequences of DUB-1, DUB-2, and DUB-2A are shown. Boxed residues are different among the various DUB proteins. The hypervariable region of the DUB-2A protein extends from amino acids 432 through 451. On the basis of this alignment, DUB-2A has a 95% identity with DUB-2 and an 86% identity with DUB-1.

conforms to a consensus sequence for a eukaryotic splice site. A region of the *DUB-2A* genomic clone, 5′ to the ATG translation start site, contains a stop codon.

We next compared the predicted amino acid sequence of DUB-2A with the previously cloned DUB-1 and DUB-2 proteins[28] (Figure 3). DUB-2A has 95% amino acid identity with DUB-2 and 86% amino acid identity with DUB-1. The DUB-2A protein contains the highly conserved C (cystein) and H (histidine) domains of other known ubps.[8,13] In addition, there is a highly conserved D residue at DUB-2A, position 133. These domains are likely to form the enzyme's active site. The putative active site nucleophile of *DUB-2A* is a cysteine residue (C60) in the C domain. In addition, *DUB-2A* contains a lysine-rich region (amino acids 74 to 84) and a short hypervariable region (amino acids 431 to 450) in which the DUB-2 sequence diverges from DUB-1 and DUB-2 (Figure 3). The hypervariable region of *DUB-2A* contains the sequence VPQEQNHQKLGQKHRNNEIL, extending from amino acid residue 432 to 451.

DUB-1, DUB-2, and DUB-2A proteins are more related to each other than to other members of the ubp family of deubiquitinating enzymes. For instance, DUB-1, DUB-2, and DUB-2A contain sequence similarity not only in the conserved C and H domains (common to all ubps), but also throughout the carboxy-terminal region of the proteins. Taken together, these results further support the existence of a DUB subfamily of ubps, as previously described.[28]

Expression pattern of the *DUB-2A* mRNA

We next used *DUB-2A*-specific PCR primers to determine the expression pattern of the *DUB-2A* mRNA (Figure 4). Using PCR from genomic *DUB* clones (indicated in Figure 2A), we demonstrated that one primer pair (exon 1 and *DUB-2A*-b) specifically amplifies the *DUB-2A* sequence (lane 1) but does not amplify the highly related *DUB-2* sequence (lane 2). We used these primer pairs and RT-PCR to examine the expression of the *DUB-2A* mRNA in various murine cell lines (lanes 3 through 7). The *DUB-2A* mRNA was expressed in CTLL (T cells), 32D (myeloid cells), and ES cells but was not expressed in F9 (carcinoma cells) or NIH3T3 (fibroblasts). The *DUB-2A* mRNA was also detected in Ba/F3 cells (data not shown). The identification of the cDNA isolated from CTLL, 32D, and ES cells was confirmed as *DUB-2A* by direct DNA sequencing of the amplified cDNA product (data not shown). As a control, degenerate *DUB* primers, capable of amplifying other *DUB* family members, yielded an amplified RT-PCR product from all murine cell lines tested. Taken together, these data demonstrate that *DUB-2A* is expressed primarily in hematopoietic cells, a pattern that is similar but not identical to the expression pattern of *DUB-2*.

The *DUB-2A* gene encodes a functional deubiquitinating enzyme

To determine whether *DUB-2A* has deubiquitinating activity, we expressed *DUB-2A* as a GST fusion protein (Figure 5). The open

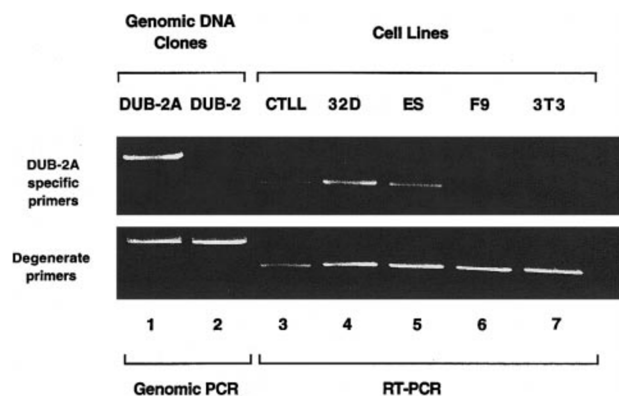

Figure 4. Expression pattern of the *DUB-2A* mRNA. Genomic PCR products and RT-PCR products were generated from the indicated cell lines, by means of degenerate primers and *DUB-2A*-specific primers. The PCR products were electrophoresed on a 1% agarose gel, which was stained with ethidium bromide. The *DUB-2A* mRNA was detected by RT-PCR in several murine cell lines. including CTLL (T cells), 32D (myeloid cells), and ES cells, but not in murine F9 carcinoma cell lines or NIH3T3 fibroblasts. A *DUB* mRNA was detected by RT-PCR from all cell lines when degenerate *DUB* primers were used (lanes 3 though 7).

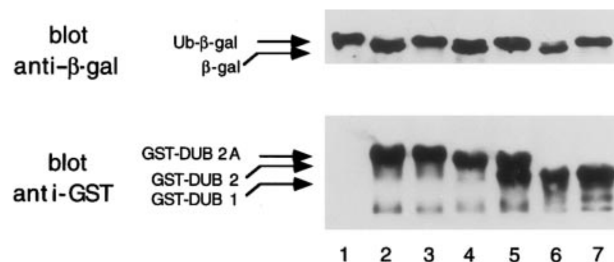

Figure 5. *DUB-2A* is a functional deubiquitinating enzyme. The upper panel shows the deubiquitination of the ubiquitin-β-galactosidase (Ub-Met-β-gal) fusion protein by various GST-*DUB* proteins coexpressed in bacteria. A Western blot using anti–β-gal antiserum is shown. Coexpressed plasmids were pBlueScript–*DUB-2A* (*DUB-2A* is not expressed) (lane 1); pGEX–*DUB-2A* (lane 2); pGEX–*DUB-2A* (C60S) (lane 3); pGEX–*DUB-2* (lane 4); pGEX–*DUB-2* (C60S) (lane 5); pGEX–*DUB-1* (lane 6); and pGEX–*DUB-1* (C60S) (lane 7). In the lower panel, GST-*DUB* fusion proteins were analyzed by an immunoblot with an anti-GST monoclonal antibody (Santa Cruz Biotechnology). *E coli* extracts were prepared from bacteria transformed with cDNAs encoding no GST fusion protein (empty vector) (lane 1); GST–*DUB-2A* (lane 2); GST–*DUB-2A* (C60S) (lane 3); GST–*DUB-2* (lane 4); GST–*DUB-2* (C60S) (lane 5); GST–*DUB-1* (lane 6); and GST–*DUB-1* (C60S) (lane 7). The lower band of the doublet in lane 5 is a degradation product of GST–*DUB-2* (C60S).

reading frame of *DUB-2A* was subcloned into the bacterial expression vector pGEX. The pGEX–*DUB-2A* was cotransformed into *E coli* (MC1061) with a plasmid encoding Ub-Met-β-gal, in which ubiquitin is fused to the NH2-terminus of β-galactosidase. As shown by immunoblot analysis, a cDNA clone encoding GST–*DUB-2A* fusion protein resulted in cleavage of Ub-Met-β-gal (lane 2) to an extent comparable to that observed with GST–*DUB-1* (lane 6) and GST–*DUB-2* (lane 4). As a control, cells transformed with the pBlueScript vector with a nontranscribed *DUB-2A* insert (lane 1) or with the pGEX vector (data not shown) failed to cleave Ub-Met-β-gal. A mutant *DUB-2A* polypeptide, containing a C60S mutation, was unable to cleave the Ub-Met-β-gal substrate (lane 3).

Taken together, these results demonstrate that *DUB-2A* has deubiquitinating enzyme activity and that C60 is critical for its thiol protease activity. An anti-GST immunoblot confirmed that the GST–DUB-1, GST–DUB-2, and GST–DUB-2A proteins were synthesized at comparable levels (Figure 5, lower blot). The difference in sizes of the DUB-1, DUB-2, and DUB-2A GST fusion proteins reflects the difference in size of these full-length DUB enzymes. Expression of full-length wild-type DUB proteins in transfected COS cells reveals that DUB-1, DUB-2, and DUB-2A are 59 kd, 62 kd, and 64 kd, respectively (data not shown).

The carboxy-terminal region of *DUB-2A* is not required for enzymatic activity

As previously described, the amino terminal region of *DUB-1, DUB-2,* and *DUB-2A* contains a putative catalytic region, consisting of C and H domains. The carboxy-terminal region contains a hypervariable region that may not be required for functional activity and that may confer substrate specificity. In order to determine the structural requirements of *DUB*-mediated deubiquitinating activity, we next generated a series of *DUB-1* and *DUB-2A* mutant polypeptides (Figure 6A) and synthesized these as GST-fusion proteins in *E coli* (Figure 6B). The mutant *DUB* polypeptides displayed differential activities in cleaving Ub-β-galactosidase in the *E coli*–based cotransformation assay. The C60 residue was required for deubiquitinating activity. In addition, mutations of the indicated D or H residue resulted in loss of activity, suggesting that these residues also play a critical role in the catalytic core of the enzyme. The carboxy-terminal 67 amino acids of *DUB-1* (B/V mutant) and the carboxy-terminal 70 amino acids of *DUB-2A* (M/H mutant) were not required for deubiquitinating activity. Taken together, these data demonstrate that the core catalytic domain of *DUB-1* and *DUB-2A* is sufficient for deubiquitinating activity.

The *DUB-2A* gene contains a cytokine-inducible enhancer element

We have previously identified a cytokine-response enhancer element of the murine *DUB-1* gene.[27] This minimal enhancer element of *DUB-1* is 112 bp in size and contains an *ets* site, 2 AP-1 sites, and 2 GATA sites. The enhancer is located approximately 1500 bp 5′ to the ATG translational start site of *DUB-1*. In an attempt to identify an enhancer region of the *DUB-2A* gene, we compared the 5′ sequences of *DUB-1* and *DUB-2A,* within the region of the *DUB-1* enhancer element. The complete 5′ region of the *DUB-2A* gene is shown (Figure 7A). A comparison of the *DUB-1* enhancer with the corresponding region of the *DUB-2A* gene is also shown (Figure 7B). Interestingly, there is considerable base-pair identity in this region of *DUB-1* and *DUB-2A,* suggesting conserved enhancer functional activity. The *DUB-2A* 5′ region contains conserved *ets*, AP1, and GATA sequences.

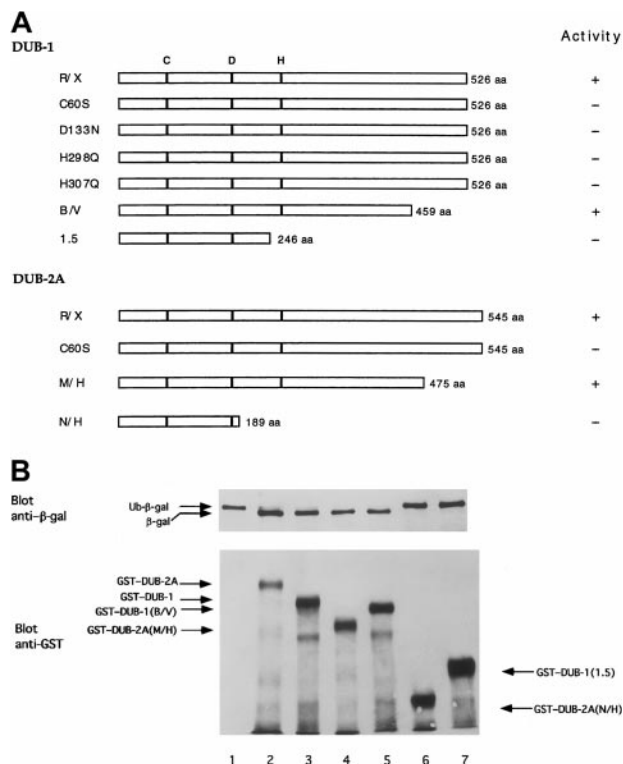

Figure 6. The carboxy-terminus of DUB enzymes is not required for enzymatic activity. (A) Schematic representation of *DUB-1* and *DUB-2A,* and mutant forms. aa indicates amino acids. (B) Deubiquitination of ubiquitin-β-galactosidase (Ub-Met-β-gal) fusion protein expressed in bacteria. The upper panel is an immuoblot using anti–β-gal antiserum. Coexpressed plasmids were pBlueScript empty vector (lane 1); pBlueScript–*DUB-2A* (lane 2); pGEX–*DUB-1* (lane 3); pGEX–*DUB-2A* (M/H) (lane 4); pGEX–*DUB-1* (B/V) (lane 5); pGEX–*DUB-2A* (N/H) (lane 6); and pGEX–*DUB-1* (1.5) (lane 7). The Ub-Met-β-gal fusion protein substrate was not cleaved in lanes 1, 6, and 7. The lower panel is an immunoblot using an anti-GST monoclonal antibody. In addtion to the full-length GST-fusion proteins, partial degradation products are also observed in lanes 2 through 7.

To assess the putative enhancer activity of the *DUB-2A* region, we next performed transfection assays in the murine hematopoietic pro-B lymphocyte cell line, Ba/F3 (Figure 7C). Ba/F3 cells are dependent on murine IL-3 for growth and survival. Ba/F3 cells were transiently transfected with various reporter constructs, and IL-3–induced *DUB-1*-luc and *DUB-2A*-luc activity were measured. The *DUB-2A* sequence, shown in Figure 7B, had an enhancer activity that was comparable to the activity of the known *DUB-1* enhancer.[27] Taken together, these data further support the notion that *DUB*s are cytokine-inducible, immediate-early gene products expressed in hematopoietic cells.

Discussion

We have previously described a family of hematopoietic-specific, cytokine-inducible immediate-early genes encoding growth-regulatory deubiquitinating enzymes (*DUB*s).[28] *DUB* mRNA is induced rapidly by cytokines, and this is followed by a rapid decline. *DUB* induction requires the activation of a cytokine receptor and a Janus kinase (JAK).[29] Sustained overexpression of *DUB* mRNA results in cell cycle arrest,[26] suggesting that these enzymes regulate cell growth by controlling the ubiquitin-dependent degradation or the ubiquitination state of a critical intracellular substrate. Several cellular proteins involved in hematopoietic cell growth, including cytokine receptors, *cbl,* and cyclin/

Figure 7. identification and functional analysis of the cytokine-inducible enhancer of the *DUB-2* gene. (A) Nucleotide sequence of the *DUB-2A* promoter. A putative TATA box (position −104) is indicated with a short underline. A 100-bp enhancer element is indicated with a long underline. In addition, a purine-rich microsatellite repeat is found between positions −560 and −385. (B) Comparison of the minimal enhancer regions of *DUB-1* and *DUB-2A.* These enhancer regions contain an ETS protein consensus sequence, 2 AP1 sites, and 3′GATA site. A CBF site, a 5′GATA site, and a TG protein–binding site are found only in *DUB-1,* as indicated. (C) Luciferase activity was assayed in Ba/F3 cells transfected with the indicated constructs. The cells were starved and restimulated with no growth factor (□) or 10 pM IL-3 (■). Luciferase assays were performed after 8 hours.

cdk inhibitors, are ubiquitinated and are potential physiologic substrates of *DUB* activity.

In the current study, we have identified a novel *DUB* enzyme, DUB-2A, which is another member of the *DUB* subfamily of deubiquitinating enzymes. DUB-2A is highly related to *DUB-2,* containing the same 2 exon genomic structure and encoding an enzyme of remarkable similarity (95% amino acid identity with *DUB-2*). *DUB-2A* is a discrete gene, however, and not an allele of the *DUB-2* gene. For instance, both *DUB-2* and *DUB-2A* genes are found in 6 independent strains of inbred mice.

Interestingly, *DUB-2A* differs from *DUB-2* and *DUB-1* in the carboxy-terminal hypervariable region, further suggesting that this region of the DUB enzyme regulates substrate specificity. For instance, this region may determine specific binding partner proteins of the various *DUB* enzymes. The existence of hypervariable regions outside of a core catalytic domain is also a feature of regulatory kinase and phosphatase enzyme families.

The *DUB* genes map to a region of murine chromosome 7 that contains a head-to-tail repeat of *DUB* genes.[28] This tandem repeat array suggests that the *DUB* genes arose by the tandem duplication of an ancestral *DUB* gene. While the tandem repeat length is unknown, recent evidence suggests a relatively short amplified unit. For instance, one of our murine BAC clones (120 kb) contains 2 complete *DUB* genes, suggesting that the amplified unit is less than 120 kb (K.-H.B., unpublished observation, December 2000). Other studies have recently identified a 4.7-kb highly repeated motif that has homology to the murine *DUB* genes.[31,32]

According to RT-PCR analysis (Figure 4) and Northern blot analysis (data not shown), the *DUB-2A* mRNA is expressed primarily in hematopoietic cells. This is similar to the expression pattern of the *DUB-2* mRNA. Interestingly, a highly related *DUB* mRNA (not *DUB-2A*) is expressed in some nonhematopoietic cell lines, such as ES cells and 3T3 fibroblasts (Figure 4), suggesting that *DUB* mRNA induction is a common feature of growth responses in other cell types as well.

While the function of the *DUB-2A* cDNA remains unknown, several features of *DUB-2A* suggest that it plays a role in hematopoietic cell growth control. First, the temporal expression of the *DUB-2A* gene is precisely regulated. The *DUB-2A* mRNA has a cytokine-inducible immediate-early pattern of expression and a

short half-life. Second, the *DUB-2A* mRNA is expressed in a precise, hematopoietic-specific pattern, suggesting that it regulates growth and differentiation of a specific subset of cellular lineages.

Several recent studies suggest that hematopoietic cell growth is regulated by ubiquitin-dependent proteolysis of critical proteins involved in cytokine signaling pathways. First, cytokine receptors have been shown to undergo ubiquitin-dependent internalization and turnover.[4,33] Second, recent evidence suggests that the mitogenic signaling protein, signal transducer and activator of transcription 1 (STAT1), is regulated, at least in part, by ubiquitin-dependent degradation.[34] Inhibition of proteasome activity also modulates signaling by the JAK/STAT pathway.[35,36] Third, other hematopoietic signaling proteins, such as cbl[37] and CIS,[38] are thought to be directly or indirectly modulated by the ubiquitin pathway. Recent studies have shown that the hematopoietic transforming protein cbl has a Ring Finger domain and is a functional E3 ubiquitin ligase.[39] Whether DUB enzymes regulate the ubiquitin-dependent degradation or the ubiquitination state of any of these hematopoietic protein substrates, leading to modulation of cellular growth, remains to be determined.

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