DUB-3, a Cytokine-inducible Deubiquitinating Enzyme That Blocks Proliferation*

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Previous studies have identified the DUB family of cytokine-regulated murine deubiquitinating enzymes, which play a role in the control of cell proliferation and survival. Through data base analyses and cloning, we have identified a human cDNA (DUB-3) that shows significant homology to the known murine DUB family members. Northern blotting has shown expression of this gene in a number of tissues including brain, liver, and muscle, with two transcripts being apparent (1.6 and 1.7 kb). In addition, expression was observed in cell lines including those derived from a number of hematopoietic tumors such as the Burkitt’s lymphoma cell line RAJI. We have also demonstrated that DUB-3, which was shown to be an active deubiquitinating enzyme, is induced in response to interleukin-4 and interleukin-6 stimulation. Finally, we have demonstrated that constitutive expression of DUB-3 blocks proliferation and can initiate apoptosis in both IL-3-dependent Ba/F3 cells and NIH3T3 fibroblasts. These findings suggest that human DUB-3, like the murine DUB family members, is transiently induced in response to cytokines and can, when constitutively expressed, block growth factor-dependent proliferation.

It has become apparent that the ubiquitin proteasome pathway plays an important role in the regulation of many cellular processes such as the cell cycle (1), transcription (2), apoptosis (3), and receptor internalization and vesicle trafficking (4, 5). This has led to attention being focused upon the proteins that modulate this pathway. These include a group of enzyme complexes, referred to as E3-ligases, as well as the E2-conjugating enzymes with which they interact to transfer ubiquitin to its targets (6). Another large group of proteins involved in this process are the deubiquitinating enzymes, a family of ubiquitin-specific proteases that cleave ubiquitin from ubiquitin-conjugated proteins and are thought to act at several points in the ubiquitin pathway, including polyubiquitin precursor processing, the removal of ubiquitin from substrates to rescue them from degradation, and the removal of residual ubiquitin to aid proteasomal degradation (7). These enzymes are classified into two main sub-families, the ubiquitin processing proteases (UBPs) and the ubiquitin carboxyl-terminal hydrolases (UCHs), which are both cysteine proteases whose active sites contain a cysteine, histidine, and aspartate residue (8). The UBPs vary greatly in size and structural complexity, but all UBPs contain six conserved homology domains (DHI–DHVI). The UCHs are a family of small, closely related proteins which lack these six domains (7). In addition to these two main sub-families, the JAMM family of metalloproteases (9), as well as the OTU family of isopeptidases (10), have recently been identified as deubiquitinating enzymes.

Although the substrates of most deubiquitinating enzymes are unknown, it is clear that they play important roles in the regulation of many cellular processes. For example, FAP (fat facets) can regulate Drosophila eye development (11), FAM (fat facets in mice) (USP9) is vital for embryonic development in mice (12), UBP3 (13) and D-ubp-64E (14) play a role in transcriptional silencing, and other UBPs have been shown to interact with important cell signaling proteins, such as p53 (15), Rb (16, 17), β-catenin (18), BRCA1 (19), and VHL (20). Deubiquitinating enzymes have also been implicated in cell transformation. In particular, a truncated isoform of the mammalian UBP, tre-2, which has no deubiquitinating activity, has been shown to transform 3T3 fibroblasts (21). Also, over-expression of unph (USP4), another mammalian UBP, induces transformation of NIH3T3 cells injected into athymic mice (22) and elevated levels of its human orthologue, unph, have been found in small cell carcinomas and adenocarcinomas of the lung (23).

The DUB family of UBPs were identified in mice as hematopoietic-specific deubiquitinating enzymes that are rapidly induced upon cytokine stimulation. DUB-1 is induced by IL-3, IL-5, and GM-CSF and is expressed in a number of hematopoietic cell types (24), whereas DUB-2 seems to be regulated specifically by IL-2, with its expression restricted to T cells (25). DUB-2A has recently been added to this family and is also primarily expressed in hematopoietic cells (26). All three of these genes are thought to form part of a head to tail repeat of DUB genes on mouse chromosome 7 that have resulted from a tandem duplication event (25). It has been suggested that the DUBs may play a role in the regulation of cell growth and survival. Both DUB-1 and DUB-2 are cytokine-inducible immediate early genes (24, 25), and high level expression of DUB-1 results in cell-cycle arrest prior to S-phase (27). Moreover, we have shown recently that DUB-2

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The abbreviations used are: UBP, ubiquitin processing protease; UCH, ubiquitin carboxyl-terminal hydrolases; HTLV-1, human T-cell lymphotropic virus I; ATL, adult T-cell leukemia; FBS, fetal bovine serum; GFP, green fluorescent protein; PI, propidium iodide; GM-CSF, granulocyte-macrophage colony-stimulating factor.
expression can markedly inhibit apoptosis induced by cytokine withdrawal (28).

Our previous work on DUB-2 originated in a study of the IL-2 pathway in cells transformed with the human T-cell lymphotropic virus I (HTLV-1). HTLV-1 is the etiologic agent for adult T-cell leukemia (ATL) (29) and is often associated with constitutive activation of the IL-2 signaling pathway (30, 31). Despite this active IL-2 signaling pathway, growth inhibitory gene products such as CIS and SOCS3 were not expressed. However, an antibody raised against murine DUB-2 cross-reacted with a band constitutively expressed in these HTLV-1-transformed T-cell lines (28). When expressed in Ba/F3 cells, DUB-2 was shown to markedly inhibit apoptosis induced by the withdrawal of cytokine (28), as well as to prolong STAT5 phosphorylation. This result indicated that DUB-2 expression could influence cell survival, possibly by modulating STAT5 activation. Interestingly, constitutive STAT activation plays an important role in many hematologic malignancies, including HTLV-1-dependent T-cell leukemia, Burkitt’s lymphoma, and myeloma, as well as a range of solid tumors. STAT activation is also required for the action of a range of oncopgenes, including src, ret, and lck (32, 33).

Here we report the identification of a human member of the DUB family of deubiquitinating enzymes (DUB-3) and show its expression in the mRNA level in a range of tissues and cell lines. In addition, we demonstrate that DUB-3, an active deubiquitinating enzyme, is induced at both the mRNA and protein levels in response to IL-4 and IL-6 stimulation. Finally, we demonstrate that DUB-3 constitutive expression blocks proliferation and leads to an increase in the number of apoptotic cells, suggesting that its expression can influence both cell proliferation and survival.

 EXPERIMENTAL PROCEDURES

Database Analysis—Basic sequence manipulations were performed with the Lasergene (DNASTAR) suite of programs. More advanced analyses were conducted through the web-based portal NIX at the UK HGMP Resource Centre.2 NIX provides an interface to multiple databases, including SwissProt, EMBL, EST and HTG, and displays the collective outputs as a simplified hyper-linked graphical representation.

RNA samples were extracted from 5 × 10⁶ Raji cells using Stat-60 reagent (Tel-Test Inc, Friendswood, TX). RT-PCR was carried out by using the OneStep RT-PCR system (Qiagen) and the primer sets D1, 5′-CAGTGAAATCTGGGGAATTGAGGACTCACTCTAC-3′, and D2, 5′-AGTCTACGCTGGCAGCAACGCTACGGC-3′. RT-PCR products were cloned by using the Perfectly Blunt cloning kit (Novagen, Madison, WI) and sequenced by using the BigDye Terminator v3.1 kit, the ABI Prism 3100 genetic analyzer (Applied Biosystems, Cheshire, UK) and the primers D3, 5′-CTCATGTTCTACGTCATGGTGCAG-3′; D4, 5′-GAGTACATCGTGGTTCAC-3′; D5, 5′-CGACATCTAGTGTTATCGCTGG-3′; D6, 5′-GGACAGAAGTGATGCT-3′; D7, 5′-TGGCAACATCATGGCTGTC-3′; and D8, 5′-CTGGAGGTTGCTCACCAAC-3′.

NORTHERN-BLASTING—Total RNA was extracted from cell lines using an RNasey midi kit (Qiagen) and quantitated by spectrophotometry. 20 μg of total RNA from each sample was subjected to electrophoresis in a 1% agarose formaldehyde gel. Gels were washed three times with water and then denatured for 30 min in 50 mM NaOH, 10 mM NaCl, rinsed with 100 mM Tris-HCl (pH 7.5) for 30 min, and placed in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min. Gels were transferred overnight onto nylon membranes (Schleicher & Schuell, Keene, NH). After transfer, membranes were rinsed with 2× SSC and cross-linked in a UV Stratalinker 2400 (Stratagene, La Jolla, CA). Membranes were prehybridized in 5 × SSC-0.5% SDS for 1 h at 65 °C and hybridized with cDNA probes by using the Rediprobe DNA labeling system (Amersham Biosciences). After hybridization, membranes were washed to a final wash in 0.1× SSC, 0.1% sodium dodecyl sulfate at 65 °C before exposure to film.

Plasmids—DUB-3 cDNAs were tagged with the FLAG epitope at their C termini by standard PCR-based methods. Each cDNA was amplified by PCR using a 5′ oligonucleotide containing an EcoRI site and an ATG codon, as well as a 3′ oligonucleotide containing a Clal site. The EcoRI/Clal PCR fragment was sub-cloned between the EcoRI and Clal sites of a modified pMIE2S vector in-frame with the FLAG epitope. To produce the catalytically inactive DUB-3C/S mutant, the cysteine residue at position 89 was changed to a serine by using the QuickChange in vitro mutagenesis kit (Stratagene). The pUHD 10–3 plasmids expressing DUB-3 were constructed by sub-cloning the DUB-3-FLAG cDNA. The pmX-IRES-EGFP plasmids expressing DUB-3 and DUB-3C/S were constructed by sub-cloning the DUB-3-FLAG and DUB-3C/S FLAG cDNAs.

Cell Culture and Transformations—The IL-3-dependent pro-B cell line Ba/F3 and Ba/F3b/TA (28) were grown in RPMI 1640 medium/10% FBS containing 10 ng/ml IL-3. RAJI and K562 cells were grown in RPMI 1640 medium/10% FBS. Platinum-B (PlATE, derived from 293T fibroblast, kind gift from Dr. T. Kitamura, University of Tokyo, Japan) and NIHET3 cells were grown in Dulbecco’s modified Eagle’s medium/10% FBS. Transfectants of Ba/F3b/TA cells were generated by electroporating 10⁷ cells with linearized pUHD10–3 plasmid containing DUB-3 using a Gene Pulsor (Bio-Rad; 300 V, 960 mF) and selected by using 1.2 μg/ml hygromycin. Tetracycline (4 μg/ml) was also added and removed every 48 h. To induce expression of DUB-3, cells were grown in the absence of tetracycline for 24–48 h. Transfectants of PlATE cells were generated by using FuGENE 6 transfection reagent (Roche Diagnostics, East Sussex, UK), as specified by the manufacturer, and either empty vector or pmX-IRES-EGFP that expresses the DUB-3-FLAG or DUB-3C/S-FLAG fusion proteins.

Antibody Production—A DUB-3 polyclonal antibody was obtained from Fusion Antibodies (Belfast, UK). Immunizations were performed by using an affinity-purified recombinant His₆-tagged fusion protein containing residues 378–530 of the DUB-3 protein.

Cell Lysis, Immunoprecipitations, and Western Blotting—To produce whole-cell lysates, cells were washed in phosphate-buffered saline and lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Brij 97, 1 mM Na₃VO₄, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 12,000 rpm at 4 °C on 10% FBS. Immunoprecipitations were carried out by using a rabbit polyclonal DUB-3 antibody (fusion antibodies) or the M2 anti-FLAG antibody (Sigma). The immunoprecipitates were then washed 5 times in lysis buffer. Cellular lysates and immunoprecipitates were separated by using SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA), and immunoblotted as appropriate by using antibodies against either DUB-3 or FLAG. The blots were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

Deubiquitinating Assay—The deubiquitinating assay, based on the cloning of ubiquitin-β-galactosidase fusion proteins, has been described previously (34). A 1590-bp fragment corresponding to the DUB-3 open reading frame (amino acids 1 to 530) and an equivalent open reading frame containing a catalytically inactive mutant form, DUB-3C/S (C98S), was generated by PCR and inserted in-frame into the pGEX vector in-frame with the glutathione S-transferase epitope. Ub-Met-β-galactosidase was expressed from a pACYC184-based plasmid. Plasmids were co-transformed into MC1061 Escherichia coli. Plasmid-bearing E. coli MC1061 cells were lysed and analyzed by immunoblotting with a rabbit anti-β-galactosidase antisera (Cappel, NC).

Growth Factor Stimulation—RAJI (IL-4), U937, and HL60 (IL-6) cells were washed and incubated for 4 h in serum-free medium. The serum-free medium, were then 20% units/ml IL-4 or IL-6 for the specified times before either cells were taken for immunoprecipitation with the DUB-3 antibody or RNA samples were extracted using the Stat-60 reagent (Tel-Test Inc) for RT-PCR. RT-PCR was carried out by using the OneStep RT-PCR system (Qiagen) with the primer sets D1, 5′-CAGTGAAATCTGGGGAATTGAGGACTCACTCTAC-3′; D2, 5′-CTCATGTTCTACGTCATGGTGCAG-3′; D4, 5′-GAGTACATCGTGGTTCAC-3′; D5, 5′-CGACATCTAGTGTTATCGCTGG-3′; D6, 5′-GGACAGAAGTGATGCT-3′; D7, 5′-TGGCAACATCATGGCTGTC-3′; and D8, 5′-CTGGAGGTTGCTCACCAAC-3′.

Northern Blotting—Total RNA was extracted from cell lines using an RNasey midi kit (Qiagen) and quantitated by spectrophotometry. 20 μg of total RNA from each sample was subjected to electrophoresis in a 1% agarose formaldehyde gel. Gels were washed three times with water and then denatured for 30 min in 50 mM NaOH, 10 mM NaCl, rinsed with 100 mM Tris-HCl (pH 7.5) for 30 min, and placed in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min. Gels were transferred overnight onto nylon membranes (Schleicher & Schuell, Keene, NH). After transfer, membranes were rinsed with 2× SSC and cross-linked in a UV Stratalinker 2400 (Stratagene, La Jolla, CA). Membranes were prehybridized in 5 × SSC-0.5% SDS for 1 h at 65 °C and hybridized with cDNA probes by using the Rediprobe DNA labeling system (Amersham Biosciences). After hybridization, membranes were washed to a final wash in 0.1× SSC, 0.1% sodium dodecyl sulfate at 65 °C before exposure to film.

2 Available on the Internet at www.hgmp.mrc.ac.uk/Registered/ Webapp/nix/.

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for the presence of GFP using EPICS, ALTRA, and EXPO software (Beckman Coulter, Buckinghamshire, UK).

Trypan Blue Exclusion Assay—Cells cultured for 12 h in medium lacking both IL-3 and serum were seeded at 2 × 10⁵ cells per ml and grown in RPMI 1640 medium/10% FBS containing 10 ng/ml IL-3 in the presence or absence of 4 μg/ml tetracycline. After the time periods identified, the numbers of viable cells were determined by standard Trypan Blue exclusion assay using a 0.4% trypan blue stain solution (Invitrogen, Paisley, UK). Cell counts were carried out in duplicate.

Propidium Iodide (PI) Staining—To assess the cell cycle profile of the selected Ba/F3 clones, they were stained with propidium iodide and analyzed by flow cytometry. Briefly, cells were washed once in phosphate-buffered saline, re-suspended in a hypotonic buffer containing 0.1% Nonidet P-40 (Sigma), 0.1% sodium citrate, and 50 mg/ml PI pressure-buffered saline, re-suspended in a hypotonic buffer containing 0.1% Nonidet P-40 (Sigma), 0.1% sodium citrate, and 50 mg/ml PI, and subjected to flow cytometry analysis using Coulter XL (Beckman Coulter).

RESULTS

Identification of Human DUB Genes—As no human members of the DUB family of deubiquitinating enzymes were known, extensive analysis of the available human EST and genomic databases was carried out by using the sequences of murine DUB-1 and DUB-2 to identify potential human family members. This procedure revealed multiple homologous genomic sequences from human chromosomes 4 and 8 (see supplementary material Fig. 1), one of which was the chromosome 4 tandem repeat RS447 that has previously been reported to contain USP17, a putative deubiquitinating enzyme (35). Using these sequences as a basis, primers were designed to allow the cloning and sequencing of full-length RT-PCR products from the RAJI cell line to identify sequences expressed at the mRNA level. Several different cDNAs were observed (results not shown), the most frequently detected of which we have designated DUB-3 (Fig. 1).

Fig. 1 shows an alignment of the protein sequences of the members of the DUB family of deubiquitinating enzymes, including murine DUB-1, DUB-2 and DUB-2A, as well as DUB-3 as a representative human member. This alignment demonstrates that DUB-3 shows 44% identity overall to the murine members of the DUB protein family, and 56% identity to the catalytic core of these enzymes, which includes the previously identified conserved domains of the UBPs (DH I–VI) as well as the cysteine, aspartate, and histidine residues conserved at their active site (7, 8). In addition, a 19-amino acid repeated sequence is highlighted (Fig. 1, broken underline) that is present three times in DUB-2/2A, twice in DUB-1, but only once in the human DUBs. The significance of this is unclear.

Tissue Distribution—To demonstrate that DUB-3 was expressed at the mRNA level, Northern blot hybridization of poly(A)+ RNAs from multiple tissues was carried out by using the full-length DUB-3 cDNA as a probe (Fig. 2A). Two transcripts were observed, one of ~1.6 kb in a number of tissues including heart, skeletal muscle, colon, kidney, and liver, with a second transcript of ~1.7 kb present in both brain and liver.

In addition, we carried out Northern blot hybridization of RNAs from a number of cell lines including those derived from a range of hematopoietic tumors such as promyelocytic leukemia, chronic myelogenous leukemia, lymphoblastic leukemia, and Burkitt's lymphoma, as well as a range of solid tumors (Fig. 2B). All but one of the cell lines examined showed high level expression of the 1.6-kb transcript. These observations contrasted with those in the multiple tissue Northern blot where normal peripheral blood leukocytes and spleen (Fig. 2A) showed negligible levels of expression.

Deubiquitinating Assay—Having cloned DUB-3, it was important to demonstrate that it was an active enzyme, and as a result, an in vitro deubiquitination assay was performed. DUB-3 was co-expressed in E. coli cells with a ubiquitin-Met-galactosidase fusion protein. Activity was assessed by the cleavage of ubiquitin from the ubiquitin-Met-galactosidase fusion protein and visualized by immunoblotting using an anti-Met-galactosidase polyclonal antibody. The results demonstrated that DUB-3 displayed deubiquitinating activity at least equivalent to that of DUB-1, and that this activity was ablated by mutating the cysteine residue (C88S) conserved within the active site which has previously been shown in other deubiquitinating enzymes to contain USP17, a putative deubiquitinating enzyme (35).

Regulation of DUB Protein Expression—To facilitate studies of the endogenous protein, a rabbit polyclonal antibody was developed against the carboxyl-terminus of DUB-3 by using a purified recombinant fusion protein containing amino acid residues 378–530, a region outside the recognized catalytic core (DHI–DHIV) (Fig. 1). The antibody was tested by using Ba/F3...
cells expressing a DUB-3-FLAG-tagged fusion protein from a TET-Off expression construct. The findings demonstrated that the antibody could immunoprecipitate and immunoblot the DUB-3 protein (Fig. 4A). These results were confirmed by immunoprecipitations and blotting using the anti-FLAG antibody M2 (data not shown).

Having demonstrated that the DUB-3 antibody recognized over-expressed DUB-3 protein, it was important to examine next whether it could recognize endogenously expressed protein. Previously, we had shown that the cell line RAJI expressed high levels of DUB-3 mRNA (Fig. 2 and results not shown). Therefore, this cell line was chosen to test for the presence of endogenous protein. Lysates from RAJI cells were immunoprecipitated by using the DUB-3 antibody and examined by Western blotting. Constitutive expression of DUB-3 protein was detected in the RAJI cell line (Fig. 4B), demonstrating that these cells expressed DUB-3 at both the mRNA and protein levels. K562 cells which did not express DUB-3 mRNA (Fig. 2B) also did not show protein expression (Fig. 4A).

DUB-1 and DUB-2 were originally identified as immediate early genes rapidly induced upon cytokine stimulation. In particular, DUB-1 has been shown to be induced by IL-3, IL-5, and GM-CSF (24), whereas DUB-2 was induced in response to IL-2 (25). To determine whether or not DUB-3 behaved in a similar manner, a study was carried out to assess its inducibility in response to IL-2, IL-4, IL-6, and granulocyte colony-stimulating factor stimulation. DUB-3 mRNA and protein levels were significantly reduced by placing cells in serum-free medium for 4 h prior to growth factor stimulation (Fig. 4, B and C). After stimulation, the mRNA expression was determined by RT-PCR using primers capable of amplifying DUB-3 and, at the protein level, by immunoprecipitation and blotting using the previously characterized DUB-3 antibody. Expression of DUB-3 was induced at the mRNA level in response to both IL-4 and IL-6 (100 units/ml) (Fig. 4B). In addition, we also demonstrated induction of DUB-3 expression at the protein level in response to IL-4 (100 units/ml) (Fig. 4C). Up-regulation of DUB-3 mRNA was observed in samples taken as little as 5 min after IL-4 treatment and maintained in both IL-4 and IL-6 for at least 30 min with the levels falling back to baseline before 90 min. In addition, to determine whether particular transcripts were responsible for this induction, the RT-PCR products obtained during these experiments were cloned, and 10 colonies were selected for sequencing. In addition to DUB-3, several different transcripts were represented, suggesting that this induction was not due to the up-regulation of a particular transcript, but to the simultaneous up-regulation of multiple DUB transcripts (data not shown). Accordingly, induction of DUB-3 protein production followed that of the mRNA, with up-regulation of the protein levels being observed after 30 min and levels not returning to baseline until after 90 min. These results suggested that, like the murine DUBs, human DUB-3 is a cytokine-inducible immediate early gene transiently expressed upon cytokine stimulation.

Expression Studies—Having identified DUB-3 as a cytokine-inducible transiently expressed protein, we next explored the functional importance of its expression. To examine this, Ba/F3 cell lines which expressed DUB-3 from a TET-Off expression system were used (Fig. 4A). To assess the effects of DUB-3 expression on proliferation, cells cultured in medium lacking IL-3 and serum for 12 h were seeded at 2 × 10^5 cells per ml and cultured in RPMI 1640 medium/10% FBS containing 10 ng/ml IL-3 in the presence and absence of tetracycline. Samples were taken every 12 h, and the numbers of viable cells were determined by using the trypan blue exclusion assay. The DUB-3-expressing clones examined showed a substantial reduction in their rate of proliferation when DUB-3 was expressed (Fig. 5A). The results shown were reproduced by using two separate DUB-3-expressing clones and are representative of five experiments. In addition, it was noted that 24–48 h after the removal of tetracycline, ~10% of the cells observed were non-viable (results

**Fig. 2. Northern blot analysis of DUB expression.** A, multiple tissue Northern blot (BD Biosciences Clontech). Two transcripts observed, one of ~1.6 kb in a number of tissues including heart, skeletal muscle, colon, kidney, and liver, and a second of ~1.7 kb in brain and liver. B, tumor cell line Northern blot. Expression of the 1.6-kb transcript observed in all the cell lines examined except K562. Expressing cell lines derived from a range of different human tumors.

**Fig. 3. DUB-3 is a functional deubiquitinating enzyme.** Extracts were prepared from *E. coli* co-transformed with a vector expressing the ubiquitin-β-galactosidase (Ub-Met-β-gal) fusion protein as well as the plasmids pGEX-2TK (no DUB expression) (lane 1); pGEX-DUB-1 (lane 2); pGEX-DUB-1(C60S) (lane 3); pGEX-DUB-3 (lane 4); and pGEX-DUB-3(C89S) (lane 5). Deubiquitinating activity was demonstrated for both DUB-1 and DUB-3 fusion proteins by the cleavage of the Ub-Met-β-gal fusion. DUB-1C60S (lane 3) and DUB-3C89S (lane 5) are catalytically inactive.
This result indicated that DUB-3 expression induced cell death in addition to slowing proliferation.

It has been observed previously that expression of DUB-1 can result in cell cycle arrest prior to S-phase (27). This finding raised the possibility that DUB-3 expression might act as a cell cycle block. To examine this possibility in more detail, cells cultured in the presence and absence of tetracycline were stained with PI to determine their cell cycle profile (Fig. 5B). Cells expressing DUB-3 showed a 50% reduction in the numbers of cells in S-phase and G2/M, suggesting cells expressing DUB-3 may not be able to exit G1. Moreover, when tetracycline was removed, ~10% of cells were observed in the sub-G1 region, indicating cells undergoing apoptosis. This finding confirmed the observations made with the trypan blue exclusion assay and indicated that expression of DUB-3 blocks proliferation and can initiate apoptosis.

To determine whether the effect of DUB-3 was cell-type specific, Ba/F3 and NIH3T3 cells were established expressing either DUB-3 or DUB-3C/S using the bicistronic vector pMX-ires-EGFP. The pMX-ires-EGFP vector expressed both the DUB-3 construct and GFP, thus allowing expressing cells to be selected for green fluorescence. The PlatE packaging cell line was transiently transfected with either pMX-ires-EGFP or pMX-ires-EGFP containing DUB-3 or DUB-3C/S, and supernatant was used to infect both Ba/F3 and NIH3T3 cells. The proportion of infected cells was then enriched by sorting cells based on GFP expression (Fig. 6A and not shown). DUB-3 and DUB-3C/S expression was confirmed by immunoprecipitation and immunoblotting using the DUB-3 antibody (Fig. 6B and not shown). The enriched cell populations, which now had a minimum of 80% of their cells infected, were cultured for 1 week, and the proportion of cells infected was reassessed by using flow cytometry. The proportion of cells expressing GFP in the populations infected with vector alone or DUB-3C/S remained within 10% of the levels following sorting. However, in both the Ba/F3 and NIH3T3 cells, the proportion of cells expressing GFP in those cell populations infected with pMX-ires-EGFP-DUB-3 dropped to <1% (Fig. 6A and not shown). These results suggested that, as observed previously, cells expressing DUB-3 proliferated significantly slower than the uninfected cells, thus allowing the uninfected

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**Fig. 4. Regulation of DUB protein expression.** A, protein lysates were extracted from 10^7 cells of K562 (chronic myelogenous leukemia) and RAJI (Burkitt's lymphoma), as well as the DUB3-expressing Ba/F3 clones 1 and 2 cultured with or without tetracycline. Lysates were immunoprecipitated and immunoblotted with DUB-3 antibody. B, RAJI (IL-4), U937, and HL60 (IL-6) cells cultured in serum-free medium for 4 h before treatment with IL-4 or IL-6 (100 units/ml) for times indicated. RT-PCR was then performed. C, protein lysates were extracted from 10^7 RAJI cells treated as in B; immunoprecipitates were prepared and treated as in A.

**Fig. 5. DUB-3 expression slows proliferation and increases the rate of apoptosis.** A, DUB3-expressing Ba/F3 cells seeded at 2 x 10^5 cells per ml and cultured with or without tetracycline were analyzed at 12-h intervals by trypan blue exclusion assay. B, DUB3-expressing Ba/F3 cells were stained using propidium iodide after 48 h with or without tetracycline and analyzed using flow cytometry.
population, which had originally only represented 10–20% of the population, to rapidly outgrow the DUB-3-expressing cells. To further confirm these observations, populations of Ba/F3 cells were infected as before, and their rate of proliferation was examined after sorting by using the trypan blue exclusion assay (Fig. 6C). Again, the cell population infected with pMX-ires-EGFP-DUB-3 grew more slowly than the control cells. These results confirmed that DUB-3 expression blocked proliferation and indicated that it was not a cell-type specific phenomenon, as it occurred in both the Ba/F3 and NIH3T3 cell lines. Importantly, it was also noted that the populations of cells expressing the catalytically inactive DUB-3C/S behaved in a manner similar to the populations infected with the vector-only control, suggesting that the effect of DUB-3 on proliferation is dependent upon its deubiquitinating activity.

DISCUSSION

In this study, we report the cloning of DUB-3, a human member of the DUB family of deubiquitinating enzymes, and show its expression at the mRNA level in a range of tissues and cell lines. We also demonstrate that DUB-3, which we have shown to be an active deubiquitinating enzyme, is induced at both the mRNA and protein levels in response to IL-4 stimulation, and that constitutive expression of DUB-3 blocks proliferation and leads to an increase in the number of apoptotic cells observed.

Prior to this report, no human members of the DUB family had been identified. However, several studies had indicated that the murine DUBs may be important for the regulation of proliferation and survival in immune cells. DUB-1 (IL-3, IL-5, GM-CSF) (24) and DUB-2 (IL-2) (25) are hematopoietic-specific immediate early genes, induced in response to cytokine stimulation, and over-expression of DUB-1 results in a cell cycle arrest prior to S-phase (27). Moreover, we have demonstrated recently that DUB-2 expression can markedly inhibit apoptosis induced in response to growth factor removal (28).

Our initial data base analyses identified a large number of highly similar sequences showing extensive homology to the murine DUBs especially within their catalytic domains. The observation of so many sequences demonstrating high levels (>95%) of identity, in conjunction with the identification of one of these sequences as a repeat (RS447), indicated that these sequences form part of a repetitive unit present on human chromosomes 4 and 8. This result also suggested that many of these sequences could represent non-functional pseudogenes. Therefore, to identify functional genes, we set about cloning those sequences expressed at the mRNA level. A number of different transcripts were observed in the RAJI cell line and in response to IL-4 stimulation, suggesting that there are multiple functional genes. However, as the high levels of similarity suggests that these transcripts encode functionally redundant proteins, we chose the most frequently observed transcript, DUB-3, for use in all further experiments.

The relationship between DUB-3 and the previously identified murine genes is unclear. The levels of homology observed, as well as the induction of DUB-3 in response to IL-4 and IL-6, would suggest it is a member of the DUB family of enzymes. However, in contrast to DUB-1, DUB-2, and DUB-2A, which has been shown previously to be primarily expressed in hematopoietic cells (24, 25, 26), DUB-3 showed little detectable expression in peripheral blood leukocytes and the spleen. Therefore, DUB-3 seems not to be hematopoietic-specific, although its induction in response to IL-4 and IL-6, as well as its expression in a number of leukemia and lymphoma cell lines would suggest that it is expressed in leukocytes in a highly regulated manner.

The regulation of DUB-3 by IL-4 and IL-6 suggested that, like its murine counterparts, it may regulate immune function. IL-4 has several roles in the immune system, including directing Th2 development and expansion (36), regulating B-cell growth and differentiation (37), secretion of IgE and IgG4 (38), and mast cell growth (39). IL-6 also plays a role in the immune system where it can promote Th2 development (40) and B-cell differentiation (41). It has also been implicated in hematopoiesis and can stimulate proliferation and differentiation of keratinocytes, hepatocytes, and nerve cells as well as stimulating the release of acute-phase proteins by hepatocytes (42). It could be hypothesized, therefore, that DUB-3 expression may well play a role in all of these processes. DUBs are not the only UBPs that play a role in the immune system. UBP43 (Usp18) has been shown to be induced in response to interferon (type 1) and lipopolysaccharide and to cleave ISG15, a ubiquitin-like
protein, from ISG-conjugated substrates (43). ISG15 is also strongly induced in response to interferon and lipopolysaccharide, and it has been proposed that ISG15 and UBP43 may combine to regulate signaling through the JAK-STAT pathway, possibly allowing them to regulate some immune responses (44). In addition, CYLD, a deubiquitinating enzyme, has recently been demonstrated to regulate activation of the NF-κB pathway by tumor necrosis factor-α family members (45).

The observation that expression of DUB-3 blocks proliferation and induces apoptosis indicated that, like the murine DUBs, it can influence proliferation and survival. It also suggests that DUB-3 acts to block the degradation of a protein involved in the negative regulation of proliferation. In particular, the failure of many cells to exit G1 would suggest that DUB-3 blocks degradation of a protein involved in the regulation of the G1/S transition.

Numerous studies have previously linked the ubiquitin-proteasome system to the control of proliferation and the cell cycle. In particular, the E3 ligase SCFskp2 has been shown to target the cyclin-dependent kinase inhibitor p27Kip1 for degradation, and the levels of skp2 fluctuate in a cell cycle-dependent manner to reduce p27Kip1 levels and allow the G1/S transition. Elevated levels of skp2 have also been reported in tumors, where it is thought to promote tumor growth by degrading p27Kip1, a known tumor suppressor (46). Also, a number of other deubiquitinating enzymes have been implicated in the control of the cell cycle. DUB-1 expression results in a block in the cell cycle prior to S-phase (27), and expression of a mutant deletion, has been shown to result in a cell cycle response to both, again possibly negating any effect upon proliferation. However, DUB-3 is only transiently expressed in response to pro-apoptotic molecule. However, the observation that expression of DUB-3 blocks proliferation and induces apoptosis (50). It is possible, therefore, that DUB-3 may only be present for a short period during the cell cycle, negating any effect upon proliferation.

Also, expression of DUB-3 was induced in response to IL-4 and IL-6. In contrast to DUB-3, the best characterized functions of these cytokines are associated with promoting growth and survival. However, DUB-3 is only transiently expressed in response to both, again possibly negating any effect upon proliferation. Also, in addition to its better characterized functions, several reports have demonstrated that DUB-4 treatment results in reduced proliferation in a number of different tumor cell lines (51–54); in one study, this was also associated with the observation of an increased number of apoptotic cells (54). This finding could suggest that the expression of DUB-3 upon IL-4 stimulation is responsible for the growth inhibition observed in these studies and that the function of IL-4 and, therefore, DUB-3 may be cell type-specific.

In conclusion, we have identified a human DUB deubiquitinating enzyme (DUB-3) which is induced in response to IL-4 and IL-6 and is expressed in a range of tissues and cell lines. We have also demonstrated that constitutive expression of DUB-3 blocks proliferation and increases the rate of apoptosis. This suggests that, like its murine counterparts, DUB-3 may well function to regulate immune responses by influencing cell proliferation and survival. However, further studies need to be carried out to identify its substrate or substrates, which will allow us to further elucidate the mechanism by which DUB-3 functions.

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