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DUB-1, a deubiquitinating enzyme with growth-suppressing activity

(cytokines/interleukin 3/ubiquitin/cell cycle)

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ABSTRACT **Cytokines regulate cell growth by inducing the expression of specific target genes. Using the differential display method, we have cloned a cytokine-inducible immediate early gene, *DUB-1* (for deubiquitinating enzyme). *DUB-1* is related to members of the UBP superfamily of deubiquitinating enzymes, which includes the oncoprotein *Tre-2*. A glutathione *S*-transferase–*DUB-1* fusion protein cleaved ubiquitin from a ubiquitin-β-galactosidase protein. When a conserved cysteine residue of *DUB-1*, required for ubiquitin-specific thiol protease activity, was mutated to serine (C60S), deubiquitinating activity was abolished. Continuous expression of *DUB-1* from a steroid-inducible promoter induced growth arrest in the G_1 phase of the cell cycle. Cells arrested by *DUB-1* expression remained viable and resumed proliferation upon steroid withdrawal. Our results suggest that *DUB-1* regulates cellular growth by modulating either the ubiquitin-dependent proteolysis or the ubiquitination state of an unknown growth regulatory factor(s).**

Interleukin 3 (IL-3) is a glycoprotein hormone that regulates growth of hematopoietic progenitor cells (1). IL-3, like other cytokines, acts during the G_1 phase of the cell cycle to drive cells into S phase. IL-3 exerts its biologic function through a specific receptor (IL-3R) that is expressed on its target cells (2, 3). The IL-3R activates multiple signal transduction pathways, including the Ras–Raf–mitogen activating protein kinase pathway and the JAK–STAT pathway, resulting in the induction of immediate early genes. How these immediate early genes couple IL-3R activation to the biochemical machinery of cell growth and cell cycle progression is poorly understood.

Cell growth and cell cycle progression are controlled, at least in part, by ubiquitin-mediated proteolysis (4, 5). Ubiquitin-mediated proteolysis requires ATP and results in covalent conjugation of target proteins with multiple ubiquitin molecules (6–9). Multiubiquitinated proteins are rapidly degraded by the 26S proteasome, a multicatalytic protease complex (10, 11). Recent evidence shows that intracellular levels of cyclins and cyclin dependent kinase inhibitors (12, 13), as well as other growth regulatory proteins, such as p53 (14, 15), c-Jun (16), and IκBα (17), are regulated by ubiquitin-mediated proteolysis. It is also possible that ubiquitination alters a protein's function without affecting its metabolic stability (18).

Little is known about the regulatory enzymes that determine which cellular proteins are specifically destroyed by ubiquitin-mediated proteolysis. Most evidence suggests that substrate specificity is determined by ubiquitin-conjugating enzymes (19, 20). Recently, a large superfamily of genes encoding deubiquitinating enzymes was identified (21). Deubiquitinating enzymes remove ubiquitin from intracellular protein conjugates by cleaving the amide linkage between the C terminus of ubiquitin and either α-amino or ε-amino groups of the substrate. These enzymes are ubiquitin specific but share certain properties with other thiol proteases. Genes for at least 15 deubiquitinating enzymes were identified from the yeast genome, making them the largest known gene family in the ubiquitin system. Several proteins implicated in growth and development, including the mammalian proteins Tre-2 and Unp and the *Drosophila* fat facets protein, were either shown to be deubiquitinating enzymes or to have sequence similarity to such enzymes (21).

In the current study, we used the strategy of differential display (22, 23) to clone an immediate early cDNA (*DUB-1*) that is specifically induced by IL-3. The *DUB-1* cDNA encodes a 526-aa protein that has deubiquitinating activity. Interestingly, misregulated expression of *DUB-1* induces cell cycle arrest in the G_1 phase of the cell cycle. Our results support the hypothesis that protein ubiquitination is important in growth-factor–mediated cellular proliferation. They also implicate deubiquitinating enzymes as regulatory enzymes that couple extracellular signaling to cell growth.

MATERIALS AND METHODS

Cells and Cell Culture. Ba/F3 is an IL-3-dependent murine pro-B cell line (24). Ba/F3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10% conditioned medium from WEHI-3B cells as a source of IL-3 (25).

Differential Display and Cloning of *DUB-1* cDNA. Total cellular RNA was isolated from starved or IL-3-stimulated Ba/F3 cells by the guanidinium isothiocyanate procedure (26) and subjected to the differential display analysis (22) (Gene Hunter, Boston). A partial cDNA fragment that was specifically induced by IL-3 was isolated using a 5′ primer (5′-TCTGTGCTGG-3′) and a 3′ primer (5′-TTTTTTTTTTT-TGT-3′) and subcloned into pCRII (Invitrogen). This partial cDNA (298 bp) was shown by dideoxy DNA sequencing to contain the 5′ and 3′ primers. A cDNA library, from Ba/F3 cells growing in IL-3, was constructed in the phage vector λZAP (Stratagene). Poly(A)$^+$ mRNA used for library construction was prepared by the Fast Track mRNA Isolation Kit (Invitrogen). The partial cDNA isolated by differential display was labeled with [^{32}P]dCTP by random prime labeling (27) and used to screen 1×10^6 plaque-forming units from the library. Three independent positive clones of different lengths that hybridized with the probe were isolated, and the corresponding plasmids were isolated from the phage clones. The longest cDNA clone was sequenced on both strands by the dideoxy DNA sequencing method (United States Biochemical).

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Abbreviations: IL-3, interleukin 3; IL-3R, IL-3 receptor; GST, glutathione *S*-transferase; ORF, open reading frame.

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Northern Blot Analysis. RNA samples (10–30 μg) were electrophoresed on denaturing formaldehyde gels and blotted onto Duralon-UV membranes (Stratagene). The cDNA inserts, purified from agarose gels (Qiagen, Chatsworth, CA), were radiolabeled (27) and hybridized for 1 hr to the filters in a 68°C oven. Hybridized filters were finally washed at room temperature in 0.1× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) and 0.1% SDS.

Deubiquitination Assay. The deubiquitination assay of ubiquitin–β-galactosidase fusion proteins has been previously described (21). A 1578-bp fragment from the wild-type *DUB-1* cDNA (corresponding to aa 1 to 526) and a cDNA containing a missense mutation (C60S) were generated by polymerase chain reaction (PCR) and inserted, in frame, into pGEX-2TK (Pharmacia) downstream of the glutathione *S*-transferase (GST) coding element. Ub–Met–βgal was expressed from a pACYC184-based plasmid. Plasmid-bearing *Escherichia coli* MC1061 cells were lysed and analyzed by immunoblotting with anti-βgal antibodies (Cappel) and the enhanced chemiluminescence system (Amersham).

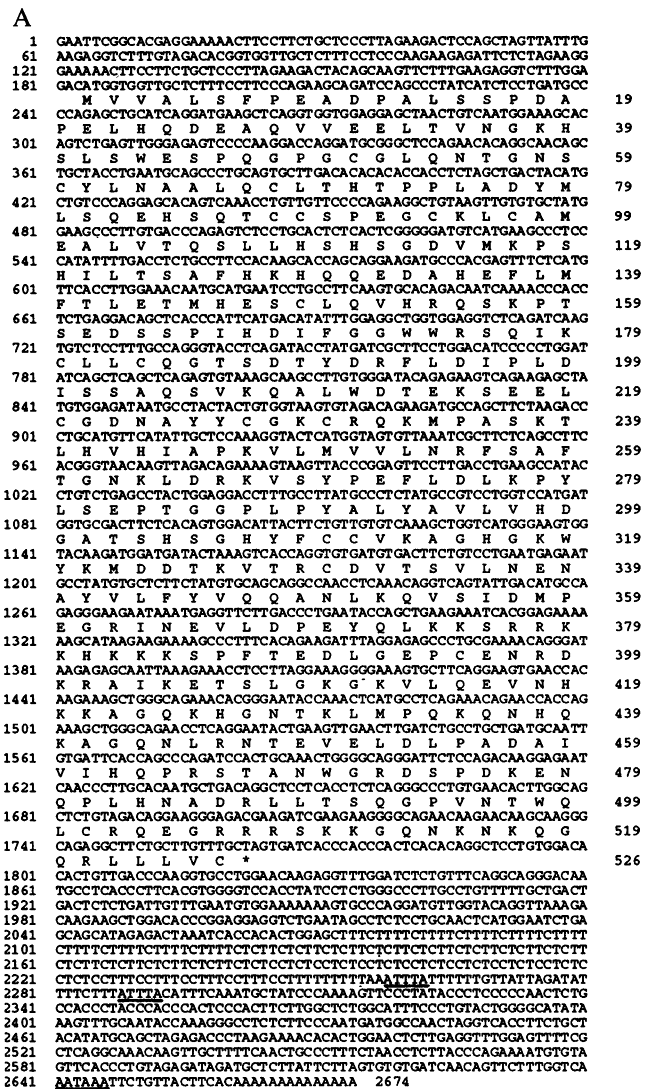
Generation of Anti-*DUB-1* Antiserum and Analysis of the *DUB-1* Polypeptide. A *DUB-1* antiserum was raised by injecting a full-length GST–*DUB-1* fusion protein into a New Zealand White rabbit and was affinity purified with a GST–DUB-1 affinity matrix, as previously described (28). *In vitro* translation of the full length *DUB-1* polypeptide was performed by standard procedures (Promega). Immunoblotting was performed as previously described (29) using the affinity-purified anti-*DUB-1* antiserum and enhanced chemiluminescence technology.

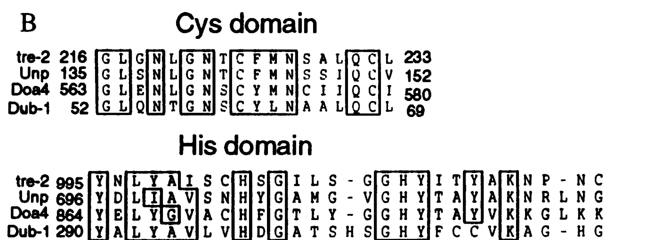
Heterologous Expression of *DUB-1* in Ba/F3 Cells and Cell Growth Analysis. The open reading frame (ORF) of *DUB-1* [or *DUB-1*(C60S)] was generated by PCR using the following primers: 5′-GCGAATTCTTTGAAGAGGTCTTTGGAGA-3′ (−19 to 1) and 5′-ATCTCGAGGTGTCCACAGGAGCCT-GTGT-3′ (1802 to 1781). The fragments (1637 bp) were subcloned into the *Sma* I/*Xho* I cloning sites of pMSG (Pharmacia), which contains a mouse mammary tumor virus–long terminal repeat inducible promoter and a *gpt* selection marker. Parental Ba/F3 cells were electroporated with vector alone or with pMSG–*DUB-1* as previously described (25). After 3 days in IL-3 medium, the cells were selected in IL-3 medium containing 250 μg/ml xanthine, 15 μg/ml hypoxanthine, 10 μg/ml thymidine, 2 μg/ml aminopterin, and 25 μg/ml mycophenolic acid. *Gpt*-resistant subclones were isolated by limiting dilution. *DUB-1* expression was induced by adding 0.1 μM dexamethasone (diluted from 10 mM stock in ethanol). Cell proliferation and cell viability were measured by trypan blue exclusion (25).

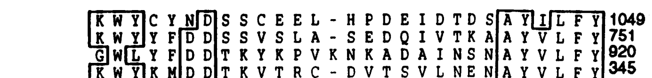
Analysis of Cell Cycle. Cell cycle analysis was performed by fluorescence-activated cell sorter, as previously described (30). The percentage of cells in each phase of the cell cycle was determined by analyzing data with the computer program CELLFIT (Becton Dickinson).

RESULTS

***DUB-1* Is a Hematopoietic-Specific Immediate Early Gene Encoding a Deubiquitinating Enzyme.** Ba/F3 is a murine lymphocyte cell line that depends on IL-3 for growth and viability (24, 30, 31). By comparing mRNA from IL-3-deprived and IL-3-stimulated Ba/F3 cells (22, 23), we initially isolated an IL-3 inducible, immediate early cDNA fragment (*DUB-1*). The full-length 2674-bp *DUB-1* cDNA was subsequently isolated and found to contain a 1581-bp ORF (Fig. 1*A*). There are two stop codons within the 183 bp of 5′ untranslated region. In addition, we isolated a murine genomic clone that contains a TATA box at position −321 and an IL-3 inducible enhancer (Y.Z., unpublished data).







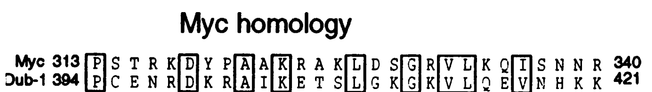


FIG. 1. Sequence and homologies of the *DUB-1* cDNA. (*A*) Nucleotide and predicted amino acid sequence of *DUB-1*. Underlined sequences are copies of a conserved motif shown by Shaw and Kamen (32) to confer message instability and which are found in the 3′ untranslated regions of many mitogen-induced, immediate early mRNAs. A consensus polyadenylylation signal is double underlined. The sequence of the murine *DUB-1* cDNA has been assigned GenBank no. 24133 U41636. (*B*) Sequence homologies between yeast Doa4 (21), human *Tre-2* (33), murine Unp (34), and murine *DUB-1*. Alignment of *DUB-1* with human c-*myc* is also shown. The homologous domain of c-*myc* contains the nuclear localization sequence PAAKRAKLD (35) but not the c-*myc* DNA binding domain.

The *DUB-1* ORF is predicted to encode a polypeptide of 526 aa (59 kDa). Comparison of the DUB-1 protein sequence with entries in GenBank data base (3/96) detected significant similarity with several deubiquitinating enzymes, including *Tre-2* (33, 36), *Unp* (34), and Doa4 (21). The sequence similarity was largely restricted to the conserved Cys and His boxes previously identified for this enzyme superfamily (Fig. 1*B*) (21). These elements probably help form the enzyme active site (21). The likely active site nucleophile is a cysteine residue in the Cys box that is found in all known family members (21) and is also present in DUB-1 (Cys60). The 3′ untranslated region of the *DUB-1* cDNA contained two ATTTA sequences, located in A + T rich domains. The AUUUA sequence, found in the 3′ untranslated regions of many immediate early mRNAs, may play a role in *DUB-1* mRNA turnover (32). The *DUB-1* mRNA was detected in multiple hematopoietic cell lines, but not in nonhematopoietic cell lines or tissues from adult mice (data not shown).

***DUB-1* Encodes a Functional Deubiquitinating Enzyme.** In order to determine whether *DUB-1* has deubiquitinating activity, we expressed *DUB-1* as a GST fusion protein. The *DUB-1* ORF was subcloned into the bacteria expression vector, pGEX. pGEX–*DUB-1* was co-transformed into *E. coli* (MC1061) with a plasmid expressing the protein Ub–Met–β gal, in which ubiquitin is fused to the N terminus of β-galactosidase. As shown by immunoblot analysis (Fig. 2), two independent cDNA clones encoding GST–*DUB-1* fusion protein resulted in cleavage of Ub–Met–β gal (lanes 3, 4, and 7) comparable to that observed with Ubp1, a known yeast deubiquitinating enzyme (21) (lane 1). As controls, cells with the pGEX vector (lane 5) or pBluescript vector with a nontranscribed *DUB-1* insert (lane 2) failed to cleave Ub–Met–β gal. A mutant *DUB-1* polypeptide, containing a C60S mutation, was unable to cleave the Ub–Met–β gal substrate (lane 6). Expression of GST-DUB-1 in bacterial cells containing the Ub–Leu–β gal substrate showed greatly reduced levels of β gal activity (data not shown). The Leu–β gal product, unlike Met–β gal or the respective Ub–β gal fusions, is short lived in *E. coli* (37). This result strongly suggests that DUB-1 cleaves Ub–Leu–β gal specifically at the C terminus of the ubiquitin moiety. Taken together, these results demonstrate that *DUB-1* has deubiquitinating activity and that Cys 60 is critical for its thiol protease activity.

***DUB-1* mRNA Levels Are Induced by IL-3 in Early G_1 Phase, Followed by a Rapid Decline.** Ba/F3 cells arrest in early G_1 phase when deprived of IL-3 for 12 hr and can be induced to reenter the cell cycle synchronously by readdition of growth factor (30). The 3.1-kb *DUB-1* mRNA appeared 30 to 60 min after addition of IL-3 (Fig. 3) but rapidly decreased in abundance before the completion of G_1 phase. *DUB-1* mRNA levels were superinduced with IL-3 plus cycloheximide (data not shown), defining *DUB-1* as an immediate early gene. Induction of *DUB-1* mRNA was similar to that of *c-myc*, although *c-myc* mRNA levels remained elevated throughout G_1 phase. Cyclin D2 mRNA accumulated later in G_1 phase as previously described (38).

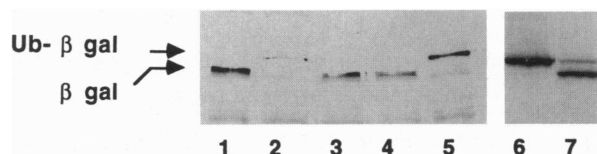
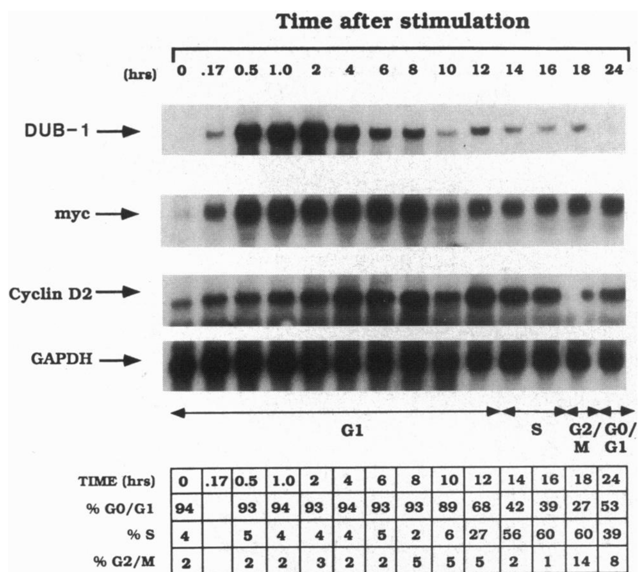


FIG. 2. *DUB-1* encodes a functional deubiquitinating enzyme. Deubiquitination of ubiquitin-β-galactosidase (Ub–Met–βgal) fusion proteins expressed in bacteria. Shown is a Western blot using anti-βgal antiserum. Co-expressed plasmids were pGEX-Ubp1 (lane 1) (21), pBluescript/DUB-1 (DUB-1 is not expressed) (lane 2), pGEX–*DUB-1*.1 (lanes 3 and 7), pGEX–*DUB-1*.2 (lane 4), pGEX(vector) (lane 5), and pGEX–*DUB-1*(C68S) (lane 6).



TIME (hrs)	0	.17	0.5	1.0	2	4	6	8	10	12	14	16	18	24
% G0/G1	94		93	94	93	94	93	93	89	68	42	39	27	53
% S	4		5	4	4	4	5	2	6	27	56	60	60	39
% G2/M	2		2	2	3	2	2	5	5	5	2	1	14	8

FIG. 3. *DUB-1* mRNA levels are induced by IL-3 in early G_1 phase, followed by a rapid decline. Ba/F3-EPO-R cells were arrested in early G_1 phase by growth factor starvation for 12 hr and were restimulated with IL-3 to enter the cell cycle synchronously. Total RNA (10 μg per lane) extracted from cells at the indicated time (in hours) was subjected to Northern blot analysis with the indicated cDNA probes. The different cell cycle phases were determined by flow-cytometric analysis of cellular DNA content.

Continuous Expression of *DUB-1* Arrests Cellular Growth. Our initial attempts to obtain stable cell lines that constitutively express *DUB-1* were unsuccessful. Because *DUB-1* expression is normally turned off after only a brief period of synthesis (Fig. 3), we reasoned that continuous expression of *DUB-1* mRNA might somehow interfere with cell growth and/or viability. We therefore expressed *DUB-1* in Ba/F3 cells using an inducible promoter (Fig. 4). Twelve *gpt*-resistant Ba/F3 subclones were generated after transfection with either pMSG/*DUB-1* or mutant pMSG/*DUB-1*(C60S), which encodes the inactive enzyme. Dexamethasone (0.1 μM) induced *DUB-1* mRNA in all transfected cells, but not in parental or mock-transfected cells (data not shown).

Dexamethasone induced expression of the DUB-1 protein (Fig. 4*A*, lane 2) or DUB-1(C60S) protein (lane 4) in transfected Ba/F3 cells to levels comparable with those observed during IL-3-induced expression from the endogenous *DUB-1* gene (data not shown). These proteins had the same electrophoretic mobility (59 kDa) as full-length *DUB-1* polypeptide synthesized by *in vitro* translation (lane 5). After dexamethasone induction, cells expressing *DUB-1* failed to proliferate in IL-3 medium (Fig. 4*B*). In contrast, dexamethasone-induced cells expressing *DUB-1* (C60S) proliferated normally in IL-3. Importantly, while dexamethasone induction of wild-type *DUB-1* inhibited cellular proliferation, as measured by total cell number, it had little effect on cellular viability (Table 1). The Ba/F3 subclones that were induced with dexamethasone to express either wild-type *DUB-1* or *DUB-1*(C60S) remained viable in IL-3. Cells underwent apoptosis only after removal of IL-3.

To test the possibility of nonspecific toxicity caused by prolonged expression of active *DUB-1* enzyme, we stopped *DUB-1* synthesis in cells transfected with the wild-type *DUB-1* construct by removal of dexamethasone at day 7. Cells resumed normal proliferation within 48 hr following dexamethasone withdrawal (Fig. 4*C*). To provide further evidence against nonspecific toxicity, we induced *DUB-1* expression in murine 3T3 fibroblasts (data not shown). Normal cell proliferation was observed for these cells, indicating that growth suppression by *DUB-1* is cell-type specific.

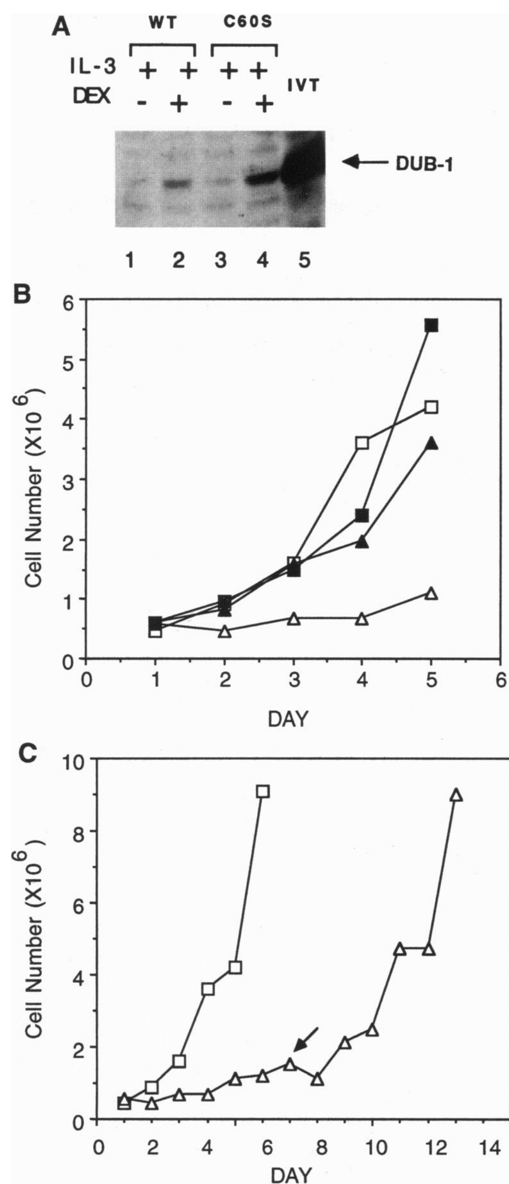


FIG. 4. Continuous expression of *DUB-1* results in growth suppression. (*A*) Immunoblot analysis of steroid-induced *DUB-1* polypeptide. Lysates (100 μg of total protein) from the indicated cells were electrophoresed in 10% SDS polyacrylamide gels and blotted with affinity-purified anti-*DUB-1* antibody (1:1000). (*B*) Ba/F3-*DUB-1* (open symbols) or Ba/F3-*DUB-1*(C60S) (filled symbols) were cultured in IL-3 medium with (△, ▲) or without (□, ■) dexamethasone (0.1 μM). Cell number was calculated by the trypan blue exclusion technique. (*C*) Ba/F3 cells, transfected with wild-type *DUB-1*, were grown in IL-3 with (△) or without (□) dexamethasone for 6 days. Dexamethasone-treated cells were washed, replated in IL-3 medium (without dexamethasone) on day 7 (arrow), and cultured for an additional 6 days (△).

Cell cycle analysis demonstrated that the majority of Ba/F3 cells were arrested in the G_1 phase of the cell cycle following dexamethasone induction of *DUB-1* (Fig. 5). This concentration of dexamethasone (0.1 μM) slightly reduced IL-3 dependent proliferation of parental Ba/F3 cells or *DUB-1*(C60S)-expressing cells (to 80% of maximum), but it completely blocked proliferation of the wild-type *DUB-1*-expressing cells.

DISCUSSION

In the present work, we describe a novel murine immediate early gene that encodes a deubiquitinating enzyme, *DUB-1*.

Table 1. Percent viable Ba/F3 cells

	IL-3	DEX	Day 0	Day 2	Day 3	Day 4
DUB-1 (WT)	+	−	96	92	88	92
	+	+	96	89	91	91
	−	−	96	0	0	0
	−	+	96	0	0	0
DUB-1 (C60S)	+	−	95	90	85	87
	+	+	95	90	80	82
	−	+	95	0	0	0
	−	−	95	0	0	0

WT, wild type; DEX, dexamethasone.

Normally, *DUB-1* mRNA levels rise soon after IL-3 addition during the early G_1 phase of the cell cycle, followed by a rapid decline. When *DUB-1* mRNA levels are maintained by continuous synthesis from a dexamethasone-inducible promoter, Ba/F3 cells arrest in the G_1 phase of the cell cycle. These data indicate that *DUB-1* expression is tightly regulated and that *DUB-1* may play a role in cytokine-induced cell proliferation.

Deubiquitinating enzymes studied in yeast have multiple functions (21). Some deubiquitinating enzymes, such as Ubp2, can apparently remove ubiquitin from ubiquitin-conjugated substrates prior to proteasome-substrate binding, thereby slowing the turnover of such proteins (39). Other deubiquitinating enzymes, such as Doa4, may remove ubiquitin from proteasome-bound degradation products, allowing recycling of ubiquitin and proteasomes and thereby promoting further protein degradation (21). Ubiquitin must also be cleaved from precursor forms by deubiquitinating enzymes. Finally, dynamic ubiquitination events may serve as reversible regulatory switches (40, 41).

Failure to turn off expression of *DUB-1* presumably, as in our experiments, may cause G_1 arrest by preventing the degradation of growth-inhibitory proteins, such as cyclin dependent kinase inhibitors, or by promoting the degradation of growth-permissive proteins, such as G_1 cyclins. Alternatively,

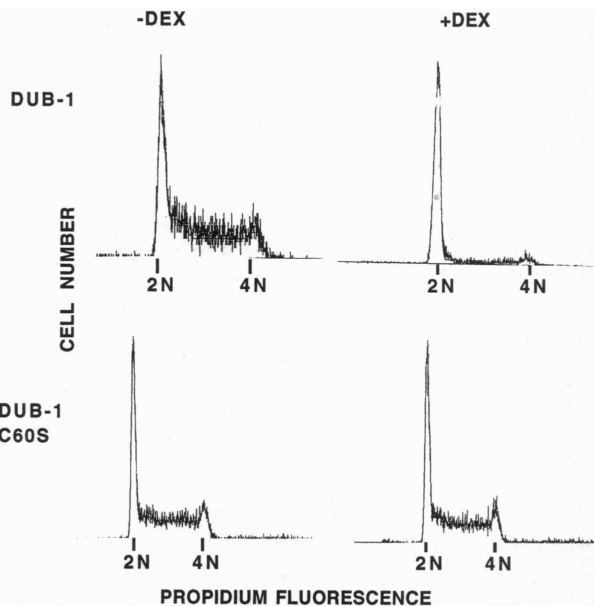


FIG. 5. Forced expression of *DUB-1* results in growth arrest in the G_1 phase of the cell cycle. The indicated cell lines were grown for 48 hr (5×10^5 cells/ml) with or without dexamethasone (0.1 μM). The cells were stained with propidium iodide and analyzed by flow cytometry. The percentage of cells in G_1, S, and G_2/M were: *DUB-1* − dexamethasone (32%, 61%, 7%), + dexamethasone (82%, 14%, 4%); *DUB-1*(C60S) − dexamethasone (35%, 57%, 8%), + dexamethasone (35%, 57%, 8%). Data shown are representative of at least three separate dexamethasone induction experiments.

DUB-1 may specifically regulate proteolysis (or the ubiquitination state) of a protein in an IL-3-specific signal transduction pathway. Identification of the specific substrates of *DUB-1* should help elucidate its mechanism of growth suppression. Constitutive expression of wild-type *DUB-1* does not suppress the growth of murine 3T3 fibroblasts (data not shown). This suggests that the growth suppression by *DUB-1* might be specific to hematopoietic cells, the only cell types in which DUB-1 is normally expressed. This may reflect the existence of a hematopoietic-specific substrate(s) of *DUB-1*, a hematopoietic cell-restricted *DUB-1* cofactor, or a higher threshold of resistance to continuous *DUB-1* expression in other cell types. Interestingly, we have isolated three additional genes whose predicted products show high sequence similarity to *DUB-1* (approximately 80% amino acid identity) (Y.Z., unpublished observations). These genes are presumably DUB subfamily members, and we refer to them as DUB-2 through DUB-4. They may be induced by different growth factors and/or may deubiquitinate different intracellular substrates.

We hypothesize that, like other immediate early gene products, *DUB-1* plays a role in integrating extracellular signals with cellular growth and cell cycle progression. Our data suggest that turning off *DUB-1* expression after a rapid burst of IL-3-induced synthesis is crucial for hematopoietic cell proliferation. It is possible that after cytokine induction, cells only turn off *DUB-1* under specific conditions, e.g., adequate nutrient availability, thereby providing a mechanism by which a cell could halt cell cycle progression following exposure to a mitogenic signal. Several other examples of mitogen-induced negative regulators have recently been demonstrated. *MKP-1* (42) and *PAC1* (43) are mitogen-induced threonine/tyrosine phosphatases that inactivate mitogen activating protein kinase. p21 is a mitogen-induced inhibitor of cyclin/cdk complexes (44). *DUB-1* is the first enzyme of the ubiquitin system directly implicated in cytokine-regulated growth control.

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