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JAK2 Is Required for Induction of the Murine *DUB-1* Gene

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Cytokine receptors activate multiple signal transduction pathways, resulting in the induction of specific target genes. We have recently identified a hematopoietic cell-specific immediate-early gene, *DUB-1*, that encodes a growth-regulatory deubiquitinating enzyme. The *DUB-1* gene contains a 112-bp enhancer element that is specifically induced by the βc subunit of the interleukin-3 (IL-3) receptor. To investigate the mechanism of *DUB-1* induction, we examined the effects of dominant-negative forms of JAK kinases, STAT transcription factors, and Raf-1 in transient transfection assays. In Ba/F3 cells, IL-3 induced a dose-dependent activation of *DUB-1*–luciferase (luc) and GAS-luc reporter constructs. A dominant-negative form of JAK2 (truncated at amino acid 829) inhibited the induction of *DUB-1*-luc and GAS-luc by IL-3. A dominant-negative form of STAT5 (truncated at amino acid 650) inhibited the induction of GAS-luc but not *DUB-1*–luc. A dominant-negative form of Raf-1 inhibited the induction of *DUB-1*–luc but had no effect on the induction of GAS-luc by IL-3. The requirement for JAK2 in the stimulation of the *DUB-1* enhancer was further supported by the suppression of *DUB-1* induction in Ba/F3 cells stably expressing the dominant-negative JAK2 polypeptide. We hypothesize that IL-3 activates a JAK2/Raf-1 signaling pathway that is required for *DUB-1* induction and is independent of STAT5.

Interleukin-3 (IL-3) is a glycoprotein hormone that regulates the growth and differentiation of hematopoietic progenitor cells (17). IL-3 activates a specific receptor (IL-3R) which is expressed on the surface of target cells. The IL-3R complex consists of an IL-3-specific α chain and a common β chain, βc, shared by the granulocyte-macrophage colony-stimulating factor receptor and the IL-5 receptor (20, 37). The IL-3R activates multiple signal transduction pathways, including the Ras/Raf/mitogen-activated protein (MAP) kinase pathway and the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) pathway, resulting in the induction of immediate-early genes. How these immediate-early genes couple receptor activation to the biochemical machinery of cell growth and cell cycle progression is poorly understood.

Discrete domains of the cytoplasmic region of the βc subunit activate the Ras/Raf/MAP kinase pathway and the JAK/STAT pathway. The distal cytoplasmic domain of βc, for example, activates the Ras/Raf/MAP kinase pathway (33, 34). This region is also required for the induction of the immediate-early genes c-*fos* and c-*jun* (34). The membrane-proximal domain of βc, in contrast, is required for activation of the JAK/STAT pathway (45).

The IL-3R, like other cytokine receptors, activates a combination of JAK and STAT proteins (7, 15). JAK kinases are cytoplasmic proteins that constitutively associate with the membrane-proximal region of cytokine receptors and become activated upon receptor-ligand interaction. STAT proteins are latent cytoplasmic transcription factors that are phosphorylated by JAK kinases. Seven STAT proteins are known at present. Phosphorylated STAT proteins form homo- and hetero-oligomeric complexes and translocate to the nucleus, where they activate transcription of specific genes. The specific set of genes induced by cytokine receptors is determined, at least in part, by the specific combination of JAK kinases and STAT proteins that are activated (16, 18).

We have recently identified an IL-3-inducible immediate-early gene, *DUB-1*, that encodes a growth-regulatory deubiquitinating enzyme (49). The *DUB-1* gene is induced by receptors that share the βc receptor subunit, including the receptors for IL-3, granulocyte-macrophage colony-stimulating factor, and IL-5. *DUB-1* is not induced by the receptor for erythropoietin (EPO) or IL-4. Furthermore, the membrane-proximal region of the βc subunit is required for *DUB-1* induction, suggesting a role for the JAK/STAT pathway (51). Interestingly, *DUB-1* is a member of a novel family of immediate-early genes encoding deubiquitinating enzymes (50). This family includes *DUB-2*, an IL-2-inducible immediate-early gene. How *DUB* genes modulate cytokine-dependent growth signals is not understood.

The mechanism by which *DUB-1* modulates IL-3-dependent growth signals remains unknown. Recent evidence implicates the ubiquitin-dependent proteolysis pathway in multiple cellular processes, including cell cycle regulation (9), transcriptional activation (38), and receptor metabolism (13). Regulatory proteins destined for degradation are initially conjugated to the 76-amino-acid peptide tag, ubiquitin. Polyubiquitinated proteins are next targeted to the proteasome, where protein degradation occurs (12). Deubiquitinating enzymes function by removing ubiquitin and thereby rescuing target proteins from degradation (14). The ubiquitin-mediated proteolytic pathway can be modified by cytokine stimulation (8). Recent evidence for ubiquitination of STAT1 (19), Cbl (40), and cytokine receptors (36) underscores the importance of ubiquitin-mediated proteolysis in signal transduction. *DUB-1* may therefore modulate cellular growth by altering the degradation or ubiquitination state of signal transducing proteins downstream of the IL-3R. In this way, the induction of DUB-1 protein may pos-

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itively or negatively feedback on IL-3-dependent signaling pathways.

We have recently delineated a minimal functional enhancer of the *DUB-1* gene that is required for βc-specific induction (51). This 112-bp enhancer includes several recognizable DNA binding elements including an ets site, two AP-1 sites, a CBF site, a TG site, and three GATA sites. To identify specific signaling pathways leading to the activation of this enhancer, we have used dominant-inhibitory forms of JAK2 kinase, STAT5, and Raf-1. Our results demonstrate that the induction of *DUB-1* requires JAK2 kinase activity and Raf-1 activity but not STAT5.

MATERIALS AND METHODS

Cells and cell culture. Ba/F3 cells are an IL-3-dependent murine pro-B cell line (28). 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 murine IL-3. For induction studies, Ba/F3 cells were washed three times in RPMI 1640 (without FCS or supplemental growth factor) and resuspended in RPMI 1640–10% FCS without IL-3. Recombinant murine IL-3 (Kirin) was used to stimulate the cells where indicated.

IL-3 and IFN-α-dependent growth characteristics of Ba/F3 cells. Ba/F3 cells, growing in IL-3-supplemented medium, were deprived of IL-3 for 8 h. Cells were next plated in 96-well microtiter plates at 2×10^5 cells/ml in the presence of variable concentrations of IL-3. For alpha interferon (IFN-α) inhibition studies, recombinant murine IFN-α (GIBCO, Gaithersburg, Md.) was added 1 h prior to the restimulation of the cells with IL-3 (0.1 pM). After an incubation period of 16 h, [^{3}H]thymidine (0.6 μCi) was added to each well and incubation continued for an additional 4 h. Afterwards, cells were harvested onto filters and washed, and incorporated [^{3}H]thymidine was quantified by liquid scintillation counting. In parallel, cell viability was assessed by the trypan blue exclusion test (54).

Immunoprecipitation analysis and immunoblotting. Immunoprecipitation and immunoblotting of proteins derived from Ba/F3 cells were performed as previously described (2), using the ECL detection system (Amersham, Arlington Heights, Ill.). Antibodies to JAK1, JAK2, and phosphotyrosine (4G10) were purchased from Upstate Biotechnology, Inc. (Lake Placid, N.Y.). Antibodies to Tyk2 and STAT5 were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Antisera to murine DUB-1 (49, 51) and to the phosphorylated form of STAT5 (1) have previously been described.

Preparation of cDNA constructs. The cDNA for wild-type murine JAK2 (WT-JAK2) was a gift from J. Ihle (45). The dominant-negative form of JAK2 (DN-JAK2), truncated at amino acid 829, was a gift from D. Wojchowski (53). The cDNAs for WT-JAK2 and DN-JAK2 were subcloned into the mammalian expression vector, pBOS (23). The cDNAs for murine wild-type STAT5A (WT-STAT5A) (10, 25) and for murine oncostatin M (OSM) (47) were gifts from J. Ihle. The cDNA for the dominant-negative form of STAT5A (DN-STAT5A) was generated by PCR, by placing a stop codon at amino acid position 650. WT-STAT5A and DN-STAT5A were tagged with the Myc epitope at the amino terminus. The cDNAs encoding the Myc-tagged WT-STAT5A and DN-STAT5A were subcloned into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, Calif.). The cDNA encoding dominant-negative Raf-1 (K373W) and wild-type Raf-1 were cloned into pLNCX and have previously been described (21). The cDNA encoding oncogenic Ras (v-Ha-Ras) was cloned in pCEP4 and has previously been described (27).

For luciferase reporter assays, the pGL2 promoter vector (Promega, Madison, Wis.) was used. pGL2 contains the luciferase gene driven by a simian virus 40 basic promoter without an enhancer. The GAS (IFN-γ activation sequence)-luc (luciferase) construct was prepared by subcloning a tandem repeat of four β-casein-like GAS elements from the β-globin locus control region (TTCTAGGAA) to a position upstream of the luciferase element. The *DUB-1*–luc construct contained the 112-bp minimal enhancer of *DUB-1* (51). The ISRE (IFN-stimulated regulatory element)-luc construct contained the endogenous enhancer of the human 2′,5′ oligo-α synthase gene (3).

Transient-transfection and transactivation experiments. All plasmid DNAs were purified by using Qiagen columns (Qiagen Inc., Chatsworth, Calif.). Ba/F3 cells were depleted of IL-3 for 8 h, washed twice in phosphate-buffered saline, and resuspended in RPMI 1640 (without serum). Cells (10^7) in RPMI 1640 (0.8 ml) were incubated with equal amounts of the indicated test plasmid, applying a total of 20 μg of DNA. Afterwards, cells and DNA were preincubated at 37°C for 15 min, followed by electroporation with a Bio-Rad electroporator (350 V, 960 μF). After electroporation, cells were incubated for an additional 10 min at 37°C before they were diluted in RPMI 1640–10% FCS, divided into pools, and cultured for 2 h in the incubator. Next, cultures were restimulated with cytokine at variable concentrations. Luciferase levels were assayed 16 h after cytokine stimulation according to the vendor's specifications (Luciferase Assay Kit; Analytical Luminescence Laboratory, San Diego, Calif.). Each construct was tested at least three times by independent transfection, with similar results each time.

Stable transfection of Ba/F3 cells with WT-JAK2 or DN-JAK2. Ba/F3 cells growing in IL-3 were transfected by electroporation as described above. The cDNA constructs applied were either pBOS (empty vector), pBOS-WT-JAK2, or pBOS-DN-JAK2 (10 μg) along with pSV2 neo (1 μg). After electroporation, cells were diluted in 20 ml of growth medium (RPMI 1640, 10% FCS, 10% WEHI conditioned medium) and grown for 48 h prior to the addition of G418 (1 mg/ml). G418-resistant Ba/F3 subclones were isolated by limiting dilution in 96-well microtiter plates. Stable expression of WT-JAK2 or DN-JAK2 was confirmed by immunoblot analysis.

Northern analysis. RNA samples (20 μg) were electrophoresed on denaturing formaldehyde gels and blotted onto Duralon-UV membranes (Stratagene, La Jolla, Calif.). The indicated cDNA inserts were purified from agarose gels (Qiagen), radiolabeled as previously described (51), and hybridized for 1 h to the membranes at 68°C. Hybridized filters were washed for 15 min in 2× SSC (0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% sodium dodecyl sulfate (SDS) and for 30 min in 0.1% SSC–0.1% SDS at room temperature.

Electrophoretic mobility shift assay. Nuclear extracts were prepared from unstimulated and IL-3-stimulated Ba/F3 cells as previously described (51). ^{32}P-labeled double-stranded DNA fragments were separated from unincorporated nucleotides with a G-50 column (Pharmacia, Uppsala, Sweden). The probe from the *DUB-1* enhancer (5′-TAACAGGAAATAATGACTAAG-3′) corresponds to nucleotides -1512 to -1494 of the *DUB-1* gene (51). A probe from the bovine β-casein promoter (5′-AGATTTCTAGGAATTCAAATC-3′) was used to analyze STAT5 binding. Binding reaction mixtures (20 μl) contained nuclear extract from 2×10^5 cells, HEPES (10 mM, pH 7.8), 65 mM NaCl, 1 mM dithiothreitol, 0.15 mM EDTA, 8% glycerol, 1 μg of poly(dI-dC), and end-labeled DNA. Binding was for 30 min on ice. For identification of STAT5 by supershift analysis, 0.5 μg of STAT5 antibody (Santa Cruz) was added afterwards, and the incubation continued for additional 20 min. In competition analysis, unlabeled DNA fragments were applied directly to the binding reaction. Samples were subjected to polyacrylamide gel electrophoresis (PAGE) on a 5% nondenaturing polyacrylamide gel, and the gel was dried and exposed to X-ray film.

RESULTS

IL-3 and IFN-α-dependent growth characteristics of Ba/F3 cells. Ba/F3 cells are an IL-3-dependent murine pro-B lymphocyte cell line (28). We initially analyzed Ba/F3 cells for IL-3-dependent and IFN-α-dependent growth characteristics (Fig. 1). IL-3 stimulated Ba/F3 cell growth within the concentration range of 0.01 to 100 pM (Fig. 1A). Half-maximal growth of Ba/F3 cells was observed at 0.1 pM, consistent with previous studies (6). IFN-α (2.2×10^6 U/mol) inhibited Ba/F3 cell growth across a concentration range of 1 to 10,000 U/ml, consistent with the known growth-suppressing effects of IFN-α (30). Half-maximal inhibition was observed at approximately 10 U of IFN-α per ml.

To further demonstrate the presence of functional IL-3 and IFN-α receptors on Ba/F3 cells, cells were stimulated with cytokine and JAK kinase activation was examined (Fig. 1B). IL-3 activated the tyrosine phosphorylation of JAK2 (Fig. 1B, top), as previously described (5, 32). IL-3 also weakly activated JAK1 tyrosine phosphorylation in these cells, though the functional relevance of JAK1 activation remains unclear (35). IFN-α activated the tyrosine phosphorylation of JAK1 and Tyk2 (Fig. 1B, bottom), as previously described (31).

Previous studies have demonstrated that IL-3 activates STAT5A and STAT5B (25), resulting in the induction of genes containing an upstream GAS consensus sequence (47, 48). Ba/F3 cells were transiently transfected with various reporter constructs, and dose-dependent luciferase activity was measured (Fig. 1C). IL-3 induced a dose-dependent increase in GAS-luc and *DUB-1*–luc activity, with half-maximal activation at approximately 0.5 pM IL-3. IL-3-induced GAS-luc and *DUB-1*–luc activity therefore correlated well with IL-3-induced mitogenesis (Fig. 1A). IFN-α induced a dose-dependent increase in ISRE-luc, with half maximal activation at approximately 1,000 U of IFN-α per ml. IFN-α-induced ISRE-luc activity therefore correlated well with IFN-α inhibition of cell growth (Fig. 1A).

JAK2 is required for the induction of the *DUB-1* gene. Previous studies have demonstrated that the IL-3R activates JAK2

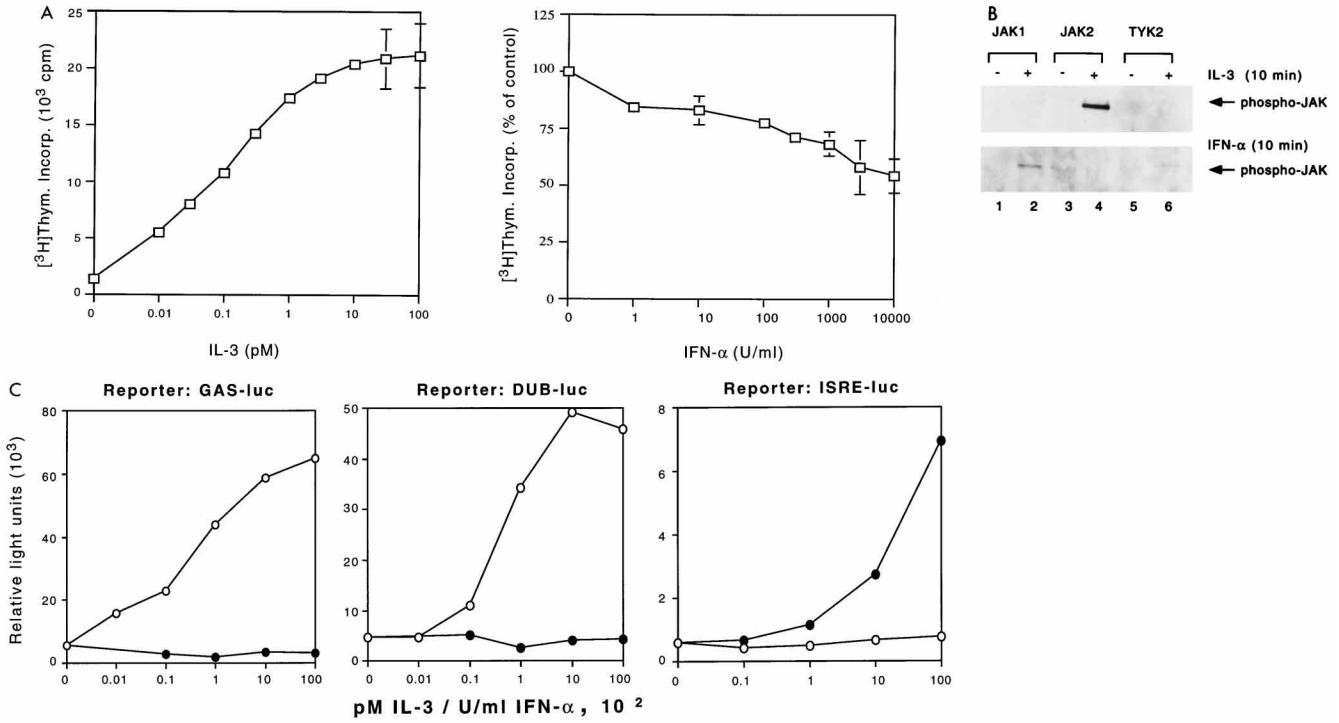

FIG. 1. IL-3- and IFN-α-dependent growth and transcriptional characterization of Ba/F3 cells. (A) Ba/F3 cells were depleted of growth factor for 8 h and stimulated with the indicated concentrations of recombinant murine IL-3. After an incubation period of 16 h, the IL-3 growth response was measured by detecting the incorporation of [^{3}H]thymidine into DNA. Values are averages ± standard deviations of triplicate experiments. Alternatively, Ba/F3 cells, cultured for 7 h without IL-3, were treated with the indicated concentrations of recombinant murine IFN-α for 1 h. IL-3 (0.1 pM) was added, and the incubation continued for an additional 16 h before the [^{3}H]thymidine incorporation assay. One hundred percent [^{3}H]thymidine incorporation corresponds to cells treated with IL-3 alone (no IFN-α). Values are averages ± standard deviations of triplicate experiments. (B) Ba/F3 cells were depleted of cytokine for 8 h and were restimulated with IL-3 at 10 pM or IFN-α at 1,000 U/ml, as indicated. Cells (10^7 per sample) were lysed, and JAK proteins were immunoprecipitated with JAK1-, JAK2-, and Tyk2-specific antibodies. Immune complexes were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a monoclonal antiphosphotyrosine antibody (4G10). (C) Ba/F3 cells were depleted of IL-3 for 8 h and were transiently transfected with the indicated luciferase reporter constructs. Transfected cells were divided into pools and incubated for 2 h before restimulation with the indicated concentration of cytokine (IL-3, open symbols; IFN-α, closed symbols). After an incubation period of 16 h, cells were lysed and luciferase activity was measured. The results shown are representative of three independent experiments.

(5, 32). A dominant-negative form of JAK2, lacking the carboxy-terminal tyrosine kinase domain, partially inhibits IL-3-induced mitogenesis (4, 52). To test the effects of DN-JAK2 on *DUB-1* induction, Ba/F3 cells were cotransfected with the cDNAs encoding either WT-JAK2 or DN-JAK2, along with various luciferase reporters (Fig. 2A). DN-JAK2 inhibited the induction of GAS-luc by IL-3, consistent with the role of JAK2 in STAT5 activation. In contrast, cotransfection with the cDNA encoding WT-JAK2 caused a small increase in GAS-luc induction. To rule out a possible nonspecific toxic effect of the JAK2 constructs, we tested the effect of DN-JAK2 on the induction of ISRE-luc. Neither WT-JAK2 nor DN-JAK2 had an effect on the induction of ISRE-luc by IFN-α. Interestingly, DN-JAK2 partially inhibited the induction of *DUB-1*–luc, demonstrating the requirement of JAK2 in the stimulation of the *DUB-1* enhancer. Again, WT-JAK2 caused a small increase, compared to transfection with the pBOS (empty) vector control.

To ensure that DN-JAK2 polypeptide was expressed in Ba/F3 cells in these transient assays, lysates from mock-transfected or DN-JAK2-transfected Ba/F3 cells were analyzed by immunoblotting (Fig. 2B). Ba/F3 cells transfected with the DN-JAK2 cDNA expressed relatively equal levels of endogenous JAK2 polypeptide and DN-JAK2 polypeptide (lane 2).

STAT5 is not required for induction of the *DUB-1* gene. IL-3 and other hematopoietic cytokines activate JAK2, which, in turn, phosphorylates STAT5A and STAT5B. Protein complexes containing STAT5 homodimers bind to GAS elements (7), found upstream of several cytokine-inducible genes (47, 48). Previous studies have demonstrated that carboxy-terminal truncations of STAT5 result in dominant inhibition of IL-3-induced mitogenesis (24, 26). Naturally occurring dominant-negative forms of STAT5A have also been identified (24, 39).

To test the potential role of STAT5 in *DUB-1* induction, we generated a deletion mutant of STAT5A, truncated at amino acid 650. Ba/F3 cells were transfected with the cDNAs encoding the Myc epitope-tagged wild-type (full-length) or truncated DN-STAT5A (Fig. 3A). DN-STAT5 inhibited activation of GAS-luc by IL-3 but did not inhibit the activation of *DUB-1*–luc by IL-3 or ISRE-luc by IFN-α. Transfection with the cDNA encoding WT-STAT5A had little effect on GAS-luc or *DUB-1*–luc. STAT5A activation is therefore not required for the induction of the *DUB-1* gene.

To ensure expression of WT-STAT5A and DN-STAT5A in transfected Ba/F3 cells, cell lysates were analyzed by anti-Myc immunoblotting (Fig. 3B). Transfected cells expressed either Myc-tagged DN-STAT5A (lane 2) or Myc-tagged WT-STAT5A (lane 3). To examine the biochemical mechanism of dominant-negative inhibition, we tested the effect of DN-STAT5A on tyrosine phosphorylation of endogenous WT-STAT5 (Fig. 3C). In the absence of IL-3 stimulation, STAT5 remained unphosphorylated (Fig. 3C, lanes 1 to 3). Following IL-3 stimulation, endogenous STAT5 was tyrosine phosphor-

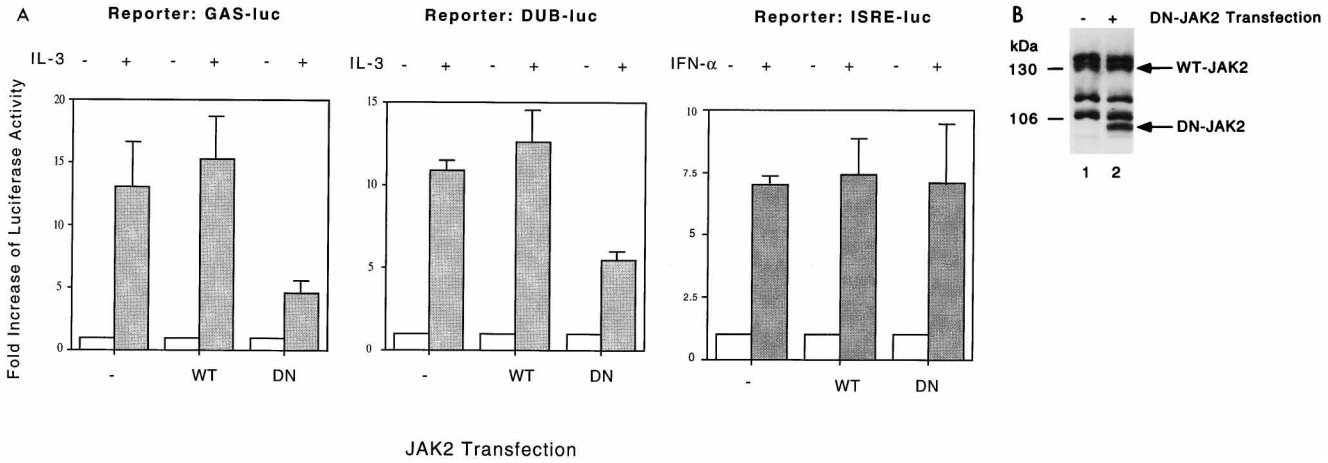

FIG. 2. JAK2 is required for induction of the *DUB-1* gene. (A) Ba/F3 cells, cultured for 8 h without IL-3, were cotransfected with the indicated luciferase reporter construct along with either pBOS (empty vector), pBOS-WT-JAK2, or pBOS-DN-JAK2. Afterwards, cells were divided into pools, stimulated with IL-3 (10 pM) or IFN-α (1000 U/ml) where indicated, and incubated for 16 h before luciferase activity was measured. To normalize for transfection efficiency, results are expressed as the fold increase of luciferase activity in stimulated versus nonstimulated cells. The data represent averages ± standard deviations of three separate transfections. (B) Cell lysates were prepared from Ba/F3 cells 16 h after transient transfection with either the empty vector (lane 1) or DN-JAK2 (lane 2). Total cell protein from the indicated transfected cells (2×10^5 per sample) was subjected to SDS-PAGE, transferred to nitrocellulose, and blotted with an anti-JAK2 antibody.

ylated in mock-transfected cells (lane 4). Cells transfected with the cDNA encoding DN-STAT5A failed to activate endogenous STAT5 (lane 5). Cells transfected with the cDNA encoding Myc-tagged WT-STAT5A demonstrated a preferential tyrosine phosphorylation of Myc-tagged STAT5 over endogenous STAT5 (lane 6), perhaps resulting from the higher expression of exogenously expressed protein.

To further analyze the effect of DN-STAT5A on *DUB-1* induction, we performed Northern blot analyses of transiently transfected Ba/F3 cells (Fig. 3D). DN-STAT5A inhibited the IL-3 induction of the murine OSM mRNA, consistent with the known function of STAT5A in OSM induction (47). DN-STAT5A had no detectable effect on the IL-3 induction of *DUB-1* or *myc*, consistent with the absence of a functional transcriptional role of STAT5A for these genes (26). We were unable to generate and confirm these results in Ba/F3 cells stably transfected with DN-STAT5A. Our subclone of Ba/F3 cells probably does not tolerate long-term expression of DN-STAT5A. Taken together, our results further support the lack of involvement of STAT5A in DUB-1 induction.

Raf-1 is required for induction of the *DUB-1* gene. Recent studies suggest a functional interaction between JAK kinases and Raf-1 (11, 43, 44, 46). To test the potential role of Raf-1 in *DUB-1* induction, a dominant-inhibitory form of Raf-1 was used (Fig. 4). DN-Raf-1 inhibited the induction of *DUB-1*–luc by IL-3. In contrast, DN-Raf-1 had no effect on the induction of GAS-luc by the cytokine, ruling out a nonspecific toxic effect of the construct. Transfection with the cDNA encoding wild-type Raf-1 caused a small increase in IL-3-dependent *DUB-1*–luc induction, whereas its effect on GAS-luc induction was insignificant. Together, these data indicate that Raf-1 contributes to the induction of the *DUB-1* gene.

To verify that JAK2 functionally interacts with Raf-1 in *DUB-1* induction, we used a constitutively activated form of Ras (v-Ha-Ras) (Fig. 5). Transient expression of v-Ha-Ras in Ba/F3 cells induced *DUB-1*–luc activity, even in the absence of IL-3. In the absence of v-Ha-Ras, DN-JAK2 inhibited IL-3-induced *DUB-1*–luc activity. When cells were transfected with both v-Ha-Ras and DN-JAK2, v-Ha-Ras partially overrode the inhibitory effect of DN-JAK2. Taken together, these results suggest that both JAK2 and the Ras/Raf-1/MAP kinase pathway contribute to *DUB-1* induction.

Analysis of stable transfectants of Ba/F3 cells expressing DN-JAK2. To further examine the role of JAK2 in the induction of the *DUB-1* gene, we generated stable Ba/F3 subclones expressing DN-JAK2 polypeptide (Fig. 6). Mock-transfected Ba/F3 cells exhibited normal IL-3-dependent growth (Fig. 6A). Ba/F3 cells expressing DN-JAK2 showed decreased IL-3-dependent growth, consistent with previous studies (52). To verify the expression and dominant-negative activity of the DN-JAK2 protein, we analyzed cell lysates from the Ba/F3 transfectants (Fig. 6B). Ba/F3 cell subclones were depleted of cytokine and restimulated with IL-3, and WT-JAK2 was analyzed for tyrosine phosphorylation. Mock-transfected cells exhibited normal IL-3-activated JAK2 phosphorylation (Fig. 6B, lane 6). DN-JAK2-expressing cells showed decreased or absent IL-3-dependent JAK2 activation (Fig. 6B, lanes 2 and 4). DN-JAK2 had no effect on the low level of IL-3-dependent JAK1 activation (data not shown).

We next tested the effects of DN-JAK2 stable expression on the IL-3 induction of DNA gel shift complexes (Fig. 7). Previous studies have shown that IL-3 activates a gel shift complex (containing tyrosine-phosphorylated STAT5) that binds to a GAS consensus sequence (25). IL-3 activates a novel gel shift complex that binds to a 20-bp sequence derived from the *DUB-1* enhancer (51). Mock-transfected Ba/F3 cells demonstrated normal IL-3-activated gel shift complexes binding a GAS consensus sequence (Fig. 7A, lane 2). This complex supershifts with an anti-STAT5 antibody (lane 4). Ba/F3 cells expressing DN-JAK2 had decreased GAS-binding complexes, consistent with diminished STAT5 activation. Interestingly, the DN-JAK2 also inhibits the formation of an IL-3-inducible gel shift complex that binds to the *DUB-1* enhancer (Fig. 7B, lane 5), confirming the importance of JAK2 in *DUB-1* induction. DN-JAK2 did not inhibit the IL-3 induction of gel shift complexes binding to an AP-1 probe (data not shown), demonstrating that the DN-JAK2 effects were specific.

JAK2 is critical for the induction of *DUB-1*. We next tested the Ba/F3 subclones expressing DN-JAK2 for induction of *DUB-1* mRNA and protein (Fig. 8). Previous studies have

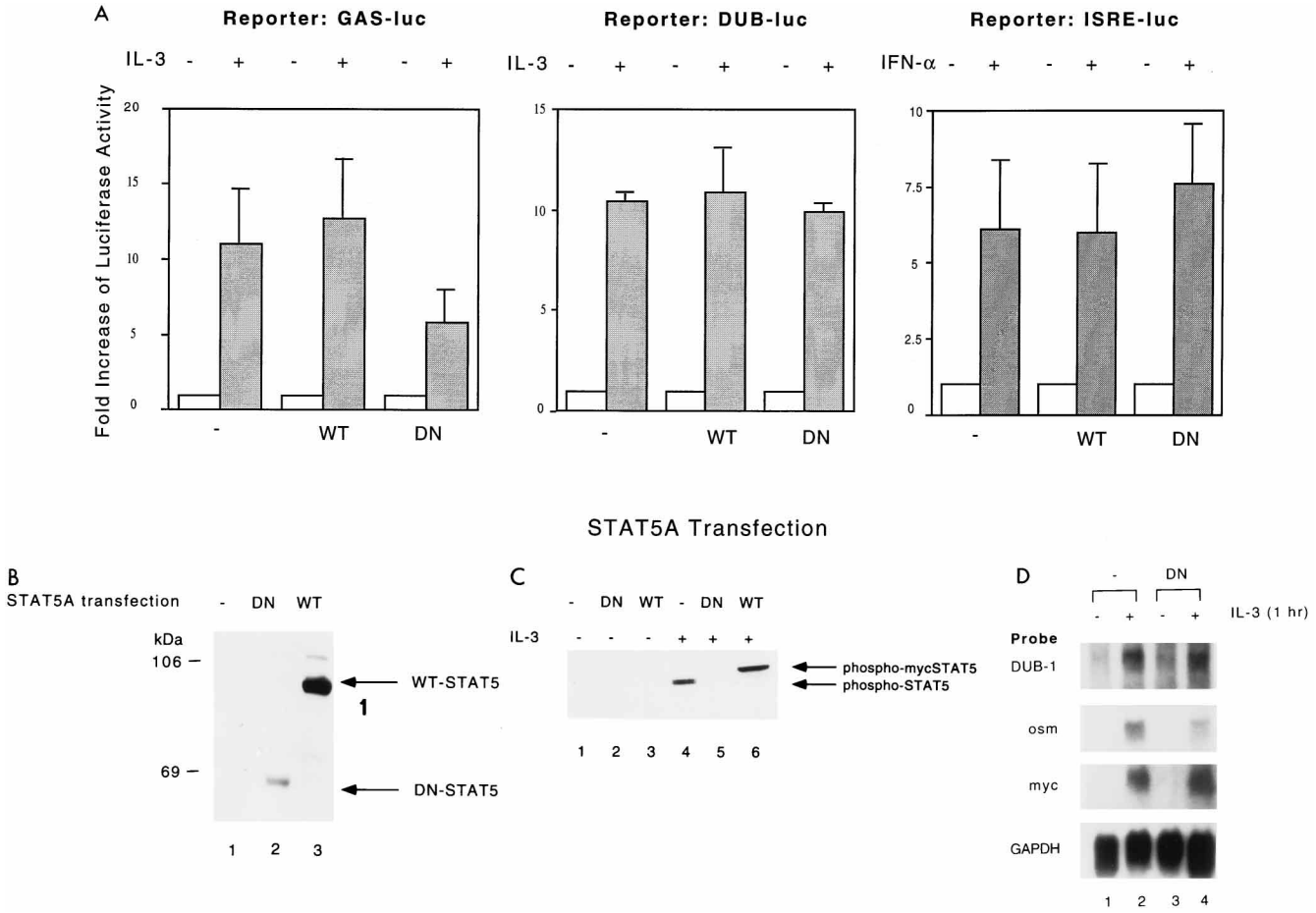

FIG. 3. STAT5 is not required for induction of the *DUB-1* gene. (A) Ba/F3 cells, depleted of growth factor for 8 h, were cotransfected with the indicated luciferase reporter construct along with either pcDNA3 (empty vector), pcDNA3-WT-STAT5A, or pcDNA3-DN-STAT5A. Afterwards, cells were divided into pools, incubated for 2 more h before cytokine stimulation, treated with IL-3 (10 pM) or IFN-α (1,000 U/ml) as indicated, and grown for 16 h before luciferase activity was measured. Results are plotted as the fold increase of luciferase activity in stimulated versus nonstimulated cells to normalize for transfection efficiency. The data are averaged values ± standard deviations of three separate transfections. (B) Transfected Ba/F3 cells (10^5 per sample) were lysed 16 h after transfection, and total cellular proteins were analyzed for *myc*-WT-STAT5 (lane WT) or *myc*-DN-STAT5 (lane DN) expression by anti-Myc immunoblotting. Lane −, empty vector. (C) Transiently transfected cells were grown for 16 h in IL-3-supplemented medium. Cells were next depleted of growth factor for 8 h, restimulated with IL-3 (10 pM) as indicated, and lysed. Total cellular proteins from 10^5 cells were resolved by SDS-PAGE and immunoblotted with a phospho-STAT5-specific polyclonal antibody. (D) Ba/F3 cells were transiently transfected with empty vector or the vector encoding DN-STAT5A. Following transfection, cells were grown for 16 h in IL-3-supplemented medium. Afterwards, cells were starved for 8 h and restimulated with IL-3 (10 pM) for 1 h. Total RNA was isolated and probed on Northern blot with a specific cDNA probe for DUB-1, murine OSM (47), Myc, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

shown that *DUB-1* is an immediate-early gene, induced by signaling pathways downstream of the IL-3R βc subunit. Three Ba/F3 subclones, stably expressing DN-JAK2, exhibited diminished IL-3 induction of *DUB-1* mRNA, consistent with the requirement of JAK2 in the *DUB-1* induction pathway (Fig. 8A, lanes 3 to 8). Stable expression of WT-JAK2 had little effect on *DUB-1* induction (Fig. 8A, lanes 9 to 12). Interestingly, DN-JAK2 did not inhibit induction of c-*myc* mRNA in these clones. To rule out the possibility of a delay in *DUB-1* induction rather than an absolute reduction, one Ba/F3 subclone expressing DN-JAK2 was induced with IL-3 over several time points (Fig. 8B). Again, DN-JAK2 inhibited *DUB-1* mRNA induction without affecting c-*myc* mRNA levels. Furthermore, c-*fos* mRNA induction was not affected in DN-JAK2-expressing cells. The inhibition of *DUB-1* expression by DN-JAK2 was also confirmed at the level of *DUB-1* protein. Ba/F3 cells stably expressing DN-JAK2 failed to induce DUB-1 polypeptide (Fig. 8C; compare lanes 2 and 4).

DISCUSSION

DUB-1 encodes a deubiquitinating enzyme and is specifically induced by the IL-3R βc subunit. In this study, we used dominant-inhibitory forms of JAK kinases and STAT proteins in order to identify signaling components required for *DUB-1* induction. We have exploited an advantage of the Ba/F3 cell system, namely, the existence of two nonoverlapping signaling pathways, one for the IL-3R and one for the IFN-α receptor. Stimulation of the IL-3R results in activation of the JAK2/STAT5 pathway; complexes containing activated STAT5 bind to GAS elements. Stimulation of the IFN-α receptor results in activation of the JAK1/Tyk2 and STAT1/STAT2 pathways; complexes containing activated STAT1, STAT2, and p48 bind to ISREs. Our results demonstrate that *DUB-1* induction requires a functional JAK2 signal.

An increasing number of immediate-early genes require JAK2. Some genes, such as the OSM gene (47), *CIS* (48), and

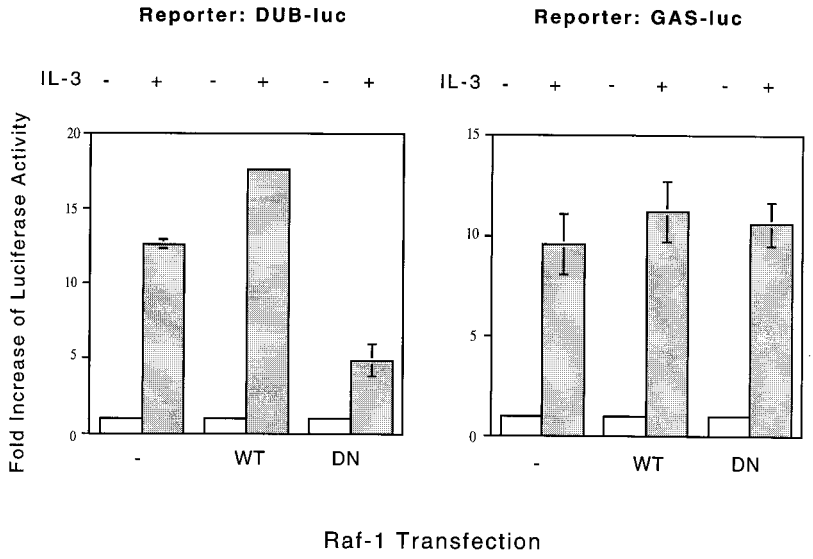

FIG. 4. Dominant-inhibitory Raf-1 inhibits the induction of *DUB-1*. Ba/F3 cells, depleted of growth factor, were cotransfected with the indicated luciferase reporter construct along with either pLNCX (empty vector), pLNCX-Raf-1, or pLNCX-Raf-1 (DN mutant). Cells were incubated for 2 more h, stimulated with IL-3 (10 pM), and analyzed for luciferase activity. Results are expressed as the fold increase of luciferase activity in stimulated versus nonstimulated cells and represent averages ± standard deviations of three separate transfections.

PIM-1 (26), require both JAK2 and STAT5. Other genes, such as c-*myc*, require JAK2 but do not require STAT5 activation (26). *DUB-1* is therefore a new member of this latter group of STAT-independent, inducible genes. *DUB-1* is the only known member of this group that modulates the ubiquitin pathway and has growth-suppressing activity (49).

Recent studies have suggested that JAK2 acts upstream of Raf-1 in signal transduction. For instance, following growth hormone stimulation, JAK2 activates the Ras/Raf/MAP kinase pathway (43, 44). Following EPO stimulation, the Ras/Raf adapter protein, Shc, is recruited to tyrosine-phosphorylated JAK2 (11). A complex of Raf-1 and JAK2 has also been identified (46), suggesting that JAK2 activates Raf-1 kinase activity directly. Our current data demonstrate that both JAK2 and Raf-1 are required for *DUB-1* induction. According to one model, the constitutively activated Ras mutant partially overrides the inhibitory effects of DN-JAK2, suggesting that JAK2 is upstream of the Ras/Raf/MAP kinase pathway in *DUB-1* induction. Alternatively, the DN-JAK2 may override the effect of activated Ras.

Activated MAP kinase is known to phosphorylate elk-1, a member of the ets family of transcription factors (29, 42). Interestingly, the minimal functional enhancer of the *DUB-1* gene contains an *ets* site. Mutation of this site ablates IL-3-dependent *DUB-1* enhancer activity (51). In contrast, mutation of the AP-1 site of the *DUB-1* enhancer only partially inhibits enhancer activity (18a). Taken together, these data suggest that elk-1, or some other ets family member, is activated by IL-3 and is required for *DUB-1* induction. The specific ets family member(s) that bind to the *DUB-1* enhancer remains unknown.

The induction of *DUB-1* differs from the induction of other JAK2-dependent immediate-early genes, c-*fos* and c-*myc* (41). In transient-transfection assays, DN-JAK2 inhibits the induction of *DUB-1*–luc, as well as *fos*-luc (data not shown), confirming the requirement of JAK2 in both *DUB-1* and *fos* transcription. Previous studies, with transient transfection assays in fibroblasts, confirm the requirement of JAK2 in βc subunit-modulated induction of c-*fos* (41). In contrast, in the stably transfected cells, DN-JAK2 inhibits *DUB-1* induction but fails to inhibit induction of c-*fos* and c-*myc* mRNAs. Taken together, these data suggest that JAK2 is more critical for *DUB-1* induction than for c-*fos* or c-*myc* induction. This observation suggests that during the selection of stable clones expressing DN-JAK2, c-*fos* and c-*myc* remain essential for cellular growth. Other kinases may substitute for JAK2 in pathways upstream of c-*myc* and c-*fos*, as previously suggested (22). The viability of DN-JAK2 stable clones lacking DUB-1 polypeptide suggests that *DUB-1* is not required for cell growth and supports a model of *DUB-1* as a growth suppressor (49).

The failure of DN-JAK2 and DN-STAT5 to block IFN-α signal transduction is consistent with previous studies. Stimu-

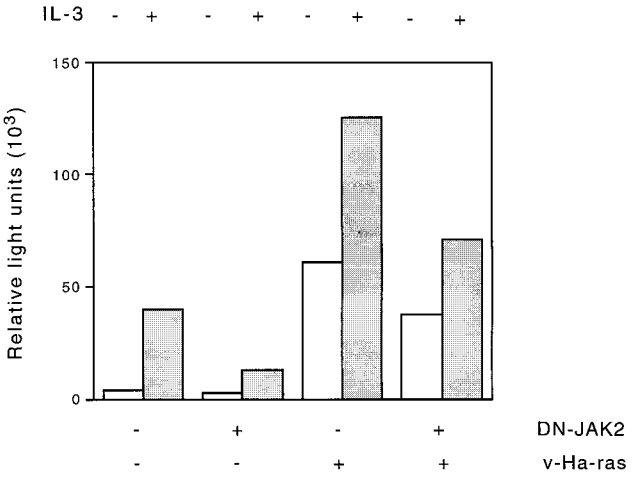

FIG. 5. JAK2 functionally interacts with the Ras/Raf-1/MAP kinase pathway, resulting in *DUB-1* induction. Ba/F3 cells were cotransfected with the *DUB-1* luciferase reporter construct, along with the cDNA for DN-JAK2 or v-Ha-Ras. Following transfection, cells were starved or stimulated with IL-3 as indicated and assayed for luciferase activity. Experiments were performed three times. The data shown are representative of all three experiments.

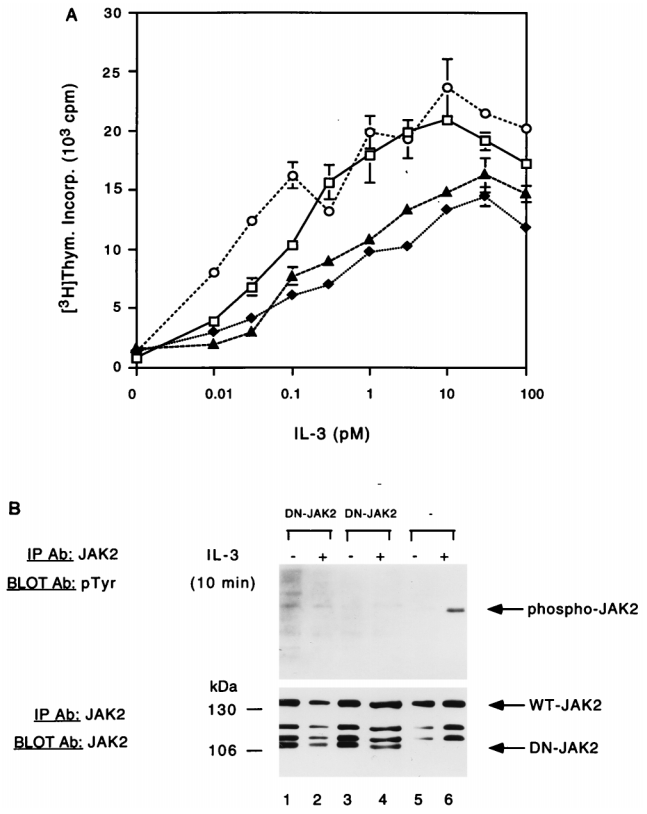

FIG. 6. Growth characteristics of Ba/F3 cells stably expressing DN-JAK2. (A) Ba/F3 cells were stably cotransfected with pSV2 neo plus pBOS (empty vector) (open symbols) or pBOS-DN-JAK2 (closed symbols). Cells were selected in G418 (1 mg/ml) and subcloned by limiting dilution. IL-3-dependent growth response of the indicated cells was quantified by detecting the incorporation of [^{3}H]thymidine into DNA as described for Fig. 1. For both transfections, the growth responses of two typical subclones are shown. Data are averaged values ± standard deviations of triplicate samples. (B) Stable Ba/F3 subclones above were analyzed for IL-3-dependent JAK2 tyrosine phosphorylation. The indicated stable cell lines (10^7 cells per sample) were cultured for 8 h in the absence of IL-3 before restimulated with the growth factor (10 pM) as indicated. Proteins were immunoprecipitated (Ip) with an anti-JAK2 antibody (Ab) (Upstate Biotechnology) and blotted with the 4G10 antiphosphotyrosine (pTyr) antibody (top). Afterwards, the blot was stripped and reprobed with anti-JAK2 (bottom). For DN-JAK2, two typical subclones are shown.

lation of the IFN-α receptor results in activation of the tyrosine kinase JAK1 and Tyk2. In our studies, DN-JAK2 had no effect on the activation of JAK1/Tyk2 pathway, implying that this pathway is independent and parallel to JAK2. Subsequently, activated Tyk2 and JAK1 tyrosine phosphorylate STAT1 and STAT2, which, in turn, interact with the ISRE (7, 15). DN-STAT5 does not disrupt this transcriptional complex.

The mechanism of βc-specific induction of *DUB-1* remains unclear. For instance, both IL-3 and EPO activate JAK2 and Ras/Raf/MAP kinase pathways. Still, *DUB-1* is selectively induced by IL-3, not by EPO. While the major pathway necessary for *DUB-1* induction is JAK2/Raf-1, this pathway is not sufficient for the specificity of *DUB-1* induction. These data suggest the existence of additional signaling pathways activated by the βc subunit but not by the EPO receptor.

The *DUB-1* gene encodes a deubiquitinating enzyme with growth-regulatory activity (49). Expression of *DUB-1* mRNA in Ba/F3 cells is highly regulated. *DUB-1* is induced as an immediate-early gene, and *DUB-1* mRNA levels rapidly decline following cytokine induction. Furthermore, DUB-1 protein levels are also highly regulated. The DUB-1 protein has a half-life of 10 min and is itself degraded by ubiquitin-mediated proteolysis (6a). Recent evidence demonstrates that multiple proteins involved in signal transduction, including growth factor receptors (36), Cbl (40), and STAT1 (19), are regulated by ubiquitin-mediated proteolysis. *DUB-1* may modulate signal transduction, by controlling the degradation or ubiquitination state of proteins required for signal transduction. Proof of this hypothesis is contingent upon the identification of specific substrates of the DUB-1 enzyme.

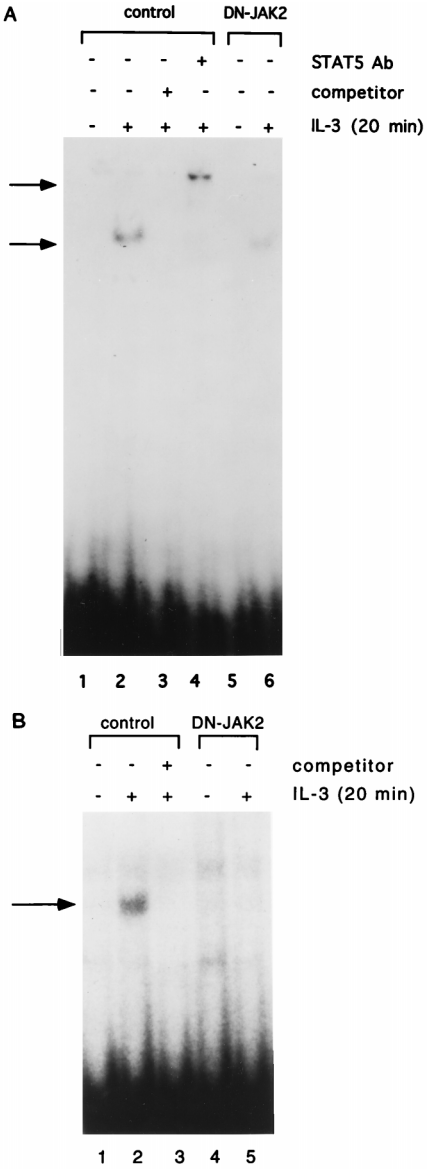

FIG. 7. DN-JAK2 inhibits the formation of gel shift complexes bound to GAS elements and *DUB-1* enhancer elements. The indicated stable Ba/F3 subclones were deprived of cytokine for 8 h and restimulated with IL-3 as indicated (10 pM). (A) Nuclear extracts were subjected to gel mobility shift analysis using a labeled β-casein GAS oligonucleotide probe. For competition analysis (lane 3), a 100-fold molar excess of the unlabeled probe was added to the binding reaction. In lane 4, supershift analysis was performed by incubating the extract with an anti-STAT5A antibody (Ab) (0.5 μg). (B) Gel mobility shift analysis was performed with a *DUB-1* enhancer probe (probe 1 from reference 51). Competition analysis (lane 3) was performed as described for panel A.

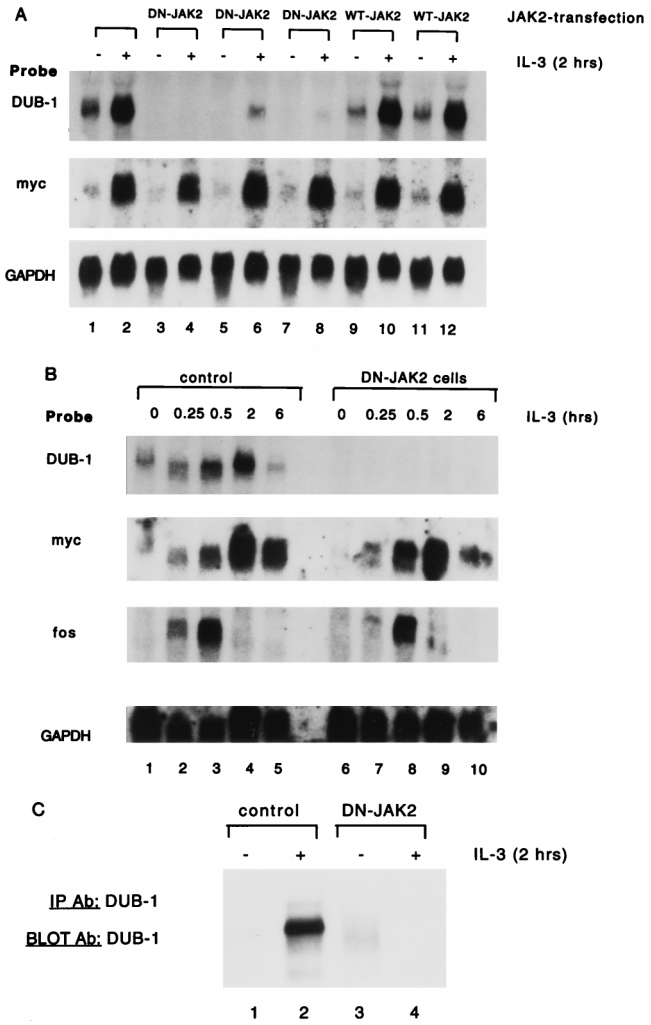

FIG. 8. DN-JAK2 inhibits the induction of *DUB-1* mRNA and DUB-1 protein. The indicated stably transfected Ba/F3 subclones were depleted of IL-3 for 8 h and restimulated with the growth factor (10 pM) as indicated (A) Northern blot analysis with the indicated ^{32}P-labeled probes. (B) Northern blot analysis with the indicated ^{32}P-labeled probes. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (C) Cellular proteins from IL-3-starved and -stimulated cells were immunoprecipitated with an anti-DUB-1 antibody (Ab) and immunoblotted with the same antibody.

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