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Analysis of *cis*-acting sequences and *trans*-acting factors regulating the interleukin-3 response element of the *DUB-1* gene

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Abstract

The murine *DUB-1* gene is a hematopoietic-specific, immediate-early gene that encodes a growth-regulatory deubiquitinating enzyme. *DUB-1* contains an IL-3-inducible enhancer element that is activated in a JAK2-dependent, STAT5-independent manner. In this study, we have further characterized this novel IL-3 response element. Transcriptional reporter assays in Ba/F3 cells revealed that two AP-1 sites, a GATA motif, and an Ets site are required for induction of *DUB-1* enhancer activity. Gel shift assays indicated that IL-3 activates the binding of an AP-1 complex containing JunD to the AP-1 sites and the binding of another protein complex to the Ets motif. The latter complex was not detectable in Ba/F3 cells stably transfected with a dominant-negative mutant of JAK2. As previously shown, these cells do not express *DUB-1* mRNA or protein. Furthermore, we demonstrated that GATA-1 constitutively binds to the *DUB-1* enhancer element. The involvement of GATA-1 may be important for the hematopoietic-restricted expression pattern of *DUB-1*. This combination of inducible and constitutive elements of the *DUB-1* enhancer appears to account for the unique STAT-independent expression characteristics of *DUB-1*.

Keywords: DUB-1; Interleukin-3; Signal transduction; AP-1; Ets; GATA

1. Introduction

Hematopoietic growth factors such as interleukin-3 (IL-3) regulate survival, growth, and differentiation of their target cells through their interaction with specific cell surface receptors [1,2]. The postreceptor signaling pathways triggered by ligand binding have been extensively studied during the past several years and are highly conserved. The IL-3 receptor (IL-3R), like other cytokine receptors, activates the Ras-Raf-MAPKinase pathway as well as a combination of Janus tyrosine kinases (JAKs) and STAT (signal transducers and activators of transcription) proteins [3,4]. The best-characterized IL-3-stimulated JAK/STAT pathway involves the tyrosine kinase JAK2 and the proteins STAT5A and STAT5B [4–6]. How receptor-activated signaling cascades are coupled to the biochemical machinery of cell proliferation and cell cycle progression is much less understood, but clearly requires the induction of specific target genes regulated through these pathways.

We have recently identified a hematopoietic-specific immediate-early gene, *DUB-1*, that encodes a growth-regulatory deubiquitinating enzyme [7,8].

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The *DUB-1* mRNA is specifically induced by IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 [8]. The receptors corresponding to these cytokines are heterodimers consisting of a specific α- and a common β-chain [9,10]. The deubiquitinating activity of the DUB-1 protein suggests an interesting link between cytokine receptor signaling, the regulation of protein degradation through ubiquitination, and cell cycle control.

We have recently identified an enhancer region of *DUB-1* (−1528 to −1416) which retains the IL-3-inducible activity of the gene [8]. This minimal IL-3 response element of *DUB-1* (IL-3 RE) contains several potential binding sites for different transcription factors including the AP-1 complex [11], Ets [12], and GATA proteins [13] as well as the core binding factor [14]. Interestingly, no STAT-binding element was observed. An analysis of the IL-3-activated signaling pathways involved in the regulation of *DUB-1* expression revealed a critical role of JAK2 and the Ras-Raf-MAPKinase pathway whereas STAT5 was not required [15]. In this study, we have further characterized the regulation of the IL-3 RE of *DUB-1*. Our data suggest that the AP-1 transcription factor JunD [16] and an Ets protein are required for the mediation of the IL-3 effect on the *DUB-1* enhancer element. Furthermore, our results implicate GATA-1 [17] in the regulation of *DUB-1* expression, providing a possible explanation for the hematopoietic-restricted expression pattern of *DUB-1*.

2. Materials and methods

2.1. Cell culture

Murine IL-3-dependent Ba/F3 cells [18] were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and IL-3 (R&D Systems, Minneapolis, MN) at 10 pM, a dose corresponding to maximum growth (data not shown). Ba/F3 cells stably expressing a dominant-negative deletion mutant of JAK2 (Δ829-JAK2) [19] were generated by cotransfection with pBOS-Δ829-JAK2 and pSV2 neo, followed by G418 selection, and have been described previously [15]. In all experiments, multiple subclones of Ba/F3 cells were tested to confirm the results.

2.2. Construction of luciferase reporter plasmids

WT and mutant *DUB-1* enhancer elements were subcloned into the PGL2Promoter plasmid (Promega) which contains the simian virus (SV) 40 basal promoter upstream from the luciferase reporter gene. The WT enhancer corresponds to the minimal IL-3 RE of the murine *DUB-1* gene (−1528 to −1416) and has previously been described [8]. Variants of the *DUB-1* IL-3 RE carrying mutations in the AP-1, GATA or CBF sites as indicated in Fig. 1 were generated by PCR using mutant oligonucleotide primers. The *DUB-1* enhancer construct with a mutant Ets site (Fig. 1) has previously been described [8]. All constructs were verified by DNA sequencing.

2.3. Transient transfections and transactivation experiments

Plasmid DNA was purified using Qiagen columns (Qiagen, Chatsworth, CA). Transient transfections of Ba/F3 cells and luciferase reporter gene assays were performed as previously described [15], with the following modifications: Ba/F3 cells were washed free of serum and IL-3 and cultured in plain RPMI for 2 h. Afterwards, they were resuspended at 1×10^7 cells per 0.8 ml RPMI and transferred to an electroporation cuvette. Cells were incubated with 10 μg of the indicated luciferase reporter vector, along with 1 μg of a cytomegalovirus promoter-driven β-galactosidase (β-gal) reporter gene construct to monitor transfection efficiencies. After electroporation with a Bio-Rad electroporator (350 V, 960 μF), cells were divided into two pools and either restimulated with IL-3 for 4 h or left untreated. Afterwards, luciferase and β-galactosidase levels were assayed according to vendor specifications (Luciferase Assay Kit, Analytical Luminescence Laboratory, San Diego, CA and Galacto-Light Kit, Tropix, Bedford, MA, respectively). Each luciferase reporter construct was tested at least three times by independent transfections.

2.4. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts from untreated and IL-3-stimulated Ba/F3 cells were prepared as previously described [20]. ^{32}P-Labeled double-stranded oligonu-

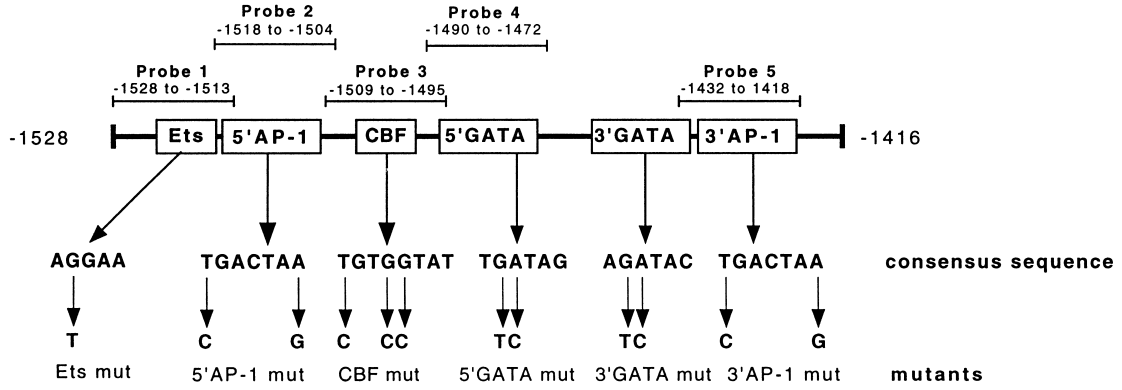

Fig. 1. Schematic representation of the IL-3 response element of *DUB-1*. The *DUB-1* enhancer is a 112 base pair fragment containing the IL-3-inducible enhancer activity of the 5′ flanking region of the gene [8]. The consensus sequences for Ets, AP-1, GATA and CBF binding, the positions of point mutations introduced into these motifs and the probes used for electrophoretic mobility shift assays are indicated.

cleotides were separated from free nucleotides using MicroSpin G-25 columns (Pharmacia, Uppsala, Sweden). The oligonucleotide probes used in these experiments were derived from the IL-3 RE of *DUB-1* and contained consensus sequences (underlined) for the binding of the following transcription factors: Ets (5′-TAACAGGAAATAATGA-3′, corresponding to nucleotides −1528 to −1513 of the *DUB-1* gene; probe 1 in Fig. 1), CBF (5′-AGACTGTGGTATGAA-3′, nucleotides −1509 to −1495; probe 3 in Fig. 1) and GATA (5′-ATTCACTGATAGTAGAAAT-3′, nucleotides −1490 to −1472; probe 4, corresponding to the 5′ GATA motif, in Fig. 1). Furthermore, two probes were designed with either the AP-1 motif next to the 5′ or the 3′ end of the IL-3 RE of *DUB-1* (probes 2 and 5 in Fig. 1). The oligonucleotide sequences are: 5′-TAATGACTAAGACTG-3′ and 5′-CAGTGACTAAGACCG-3′, corresponding to nucleotides −1518 to −1504 and −1428 to −1414 of the *DUB-1* gene, respectively. Binding reactions and competition analysis were performed essentially as described [15]. Antibodies used to identify proteins in gel-shift complexes were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Samples were electrophoresed on a 6% non-denaturating polyacrylamide gel, and the dried gel was exposed to X-ray film.

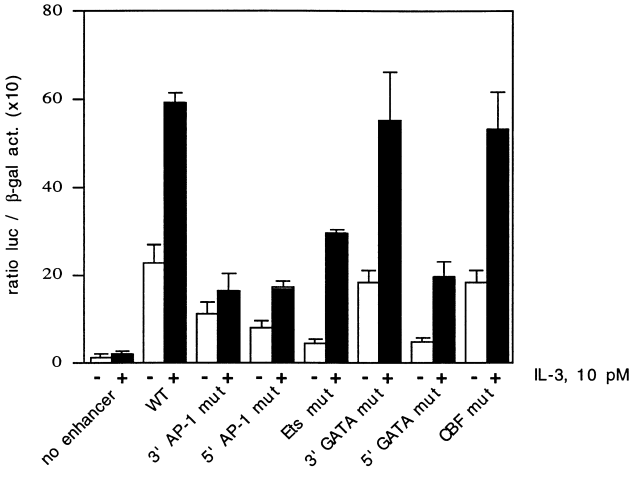

Fig. 2. Effects of mutations in the consensus sequences for AP-1, Ets, GATA and CBF binding on the activity of the *DUB-1* enhancer element. Ba/F3 cells, cultured for 2 h without IL-3 and FCS, were cotransfected with the indicated luciferase (luc) reporter constructs and a β-galactosidase (β-gal) reporter vector. The WT enhancer construct contains the minimal IL-3 RE of *DUB-1* [8]. The other luciferase reporter vectors carried the point mutations indicated in Fig. 1 in either the 5′ AP-1, 3′ AP-1, Ets, CBF, 5′ GATA or 3′ GATA motif of the WT enhancer element. The construct without enhancer element corresponds to the PGL2Promoter plasmid. After transfection, cells were divided into two pools and grown for 4 h in the absence (open bars) or presence (closed bars) of IL-3 (10 pM) before luciferase and β-galactosidase activities were measured. To normalize for transfection efficiencies, results are expressed as the ratio of luciferase to β-galactosidase activities. The data represent averages ± S.D. of three separate transfections.

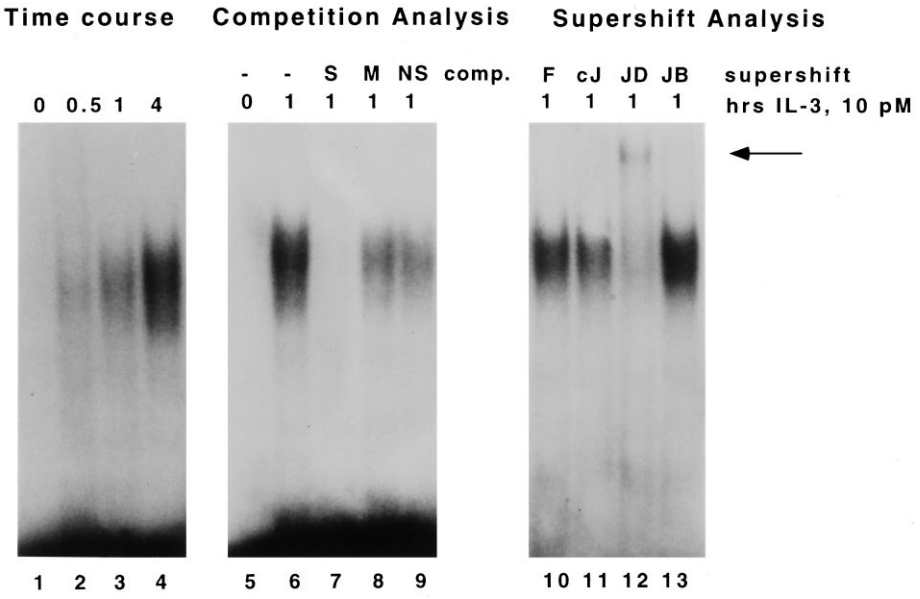

Fig. 3. IL-3 induces the binding of a protein complex containing JunD to the 5′ AP-1 site of the *DUB-1* enhancer element. Ba/F3 cells were deprived of cytokine for 8 h and restimulated with IL-3 (10 pM) for 1 h (middle and right panels) or as indicated (left panel). Nuclear extracts were subjected to gel mobility shift analysis using a ^{32}P-labeled oligonucleotide probe derived from the IL-3 RE of *DUB-1* which contains the 5′ AP-1 site (probe 2 in Fig. 1). For competition analysis (middle panel), a 100-fold molar excess of unlabeled specific probe (S, lane 7), a mutant probe carrying the point mutations indicated in Fig. 1 in the AP-1 site (M, lane 8) or a non-specific probe (NS, lane 9) was added to the binding reactions. Supershift analysis (left panel) was performed by incubating the binding reactions with antibodies (ab) (0.5 μg) against pan-Fos (F, lane 10), c-Jun (cJ, lane 11), JunD (JD, lane 12) and JunB (JB, lane 13) for an additional 20 min. The position of the complex shifted by the JunD antibody is indicated by an arrow.

3. Results

3.1. AP-1, Ets, and GATA elements are required for DUB-1 enhancer activity

In order to analyze the functional significance of potential binding sites for *trans*-acting factors, we introduced point mutations into the two consensus sequences for AP-1 binding (referred to as the 5′ and the 3′ AP-1 site), the CBF sequence, and two GATA motifs (referred to as the 5′ and the 3′ GATA site) of the IL-3 RE of *DUB-1* (Fig. 1). Of the two potential GATA sites, only the 5′ GATA motif completely matches the conventional consensus sequence for GATA binding (WGATAR), but GATA proteins have been shown to be capable of binding a variety of motifs that deviate from this sequence [21], including the AGATAC sequence of the 3′ GATA motif (Fig. 1). The enhancer activities of WT and mutant IL-3 response elements, including an Ets site mutant previously described [8] (Fig. 1), were tested by transcriptional reporter assays in Ba/F3 cells (Fig. 2). The data indicate that mutations in either of the two AP-1 motifs, the 5′ GATA site, or the Ets motif strongly suppress the enhancer activity of the *DUB-1* IL-3 RE, implicating them as important *cis*-regulatory sequences of the *DUB-1* gene. Interestingly, mutations in the AP-1 sites or the 5′ GATA motif caused the strongest inhibition of the IL-3-dependent enhancer activity. In contrast, the constitutive (non-IL-3-inducible) enhancer activity was most efficiently suppressed by a disruption of the 5′ GATA or the Ets site. Enhancer elements carrying point mutations in the CBF consensus sequence or the 3′ GATA motif displayed an IL-3-inducible enhancer activity similar to the WT IL-3 RE, suggesting that these sites are not involved in the regulation of *DUB-1* enhancer activity.

3.2. Identification of specific proteins bound to the IL-3 response element of DUB-1

To study protein binding to the IL-3 RE of *DUB-1*, we employed electrophoretic mobility shift assays.

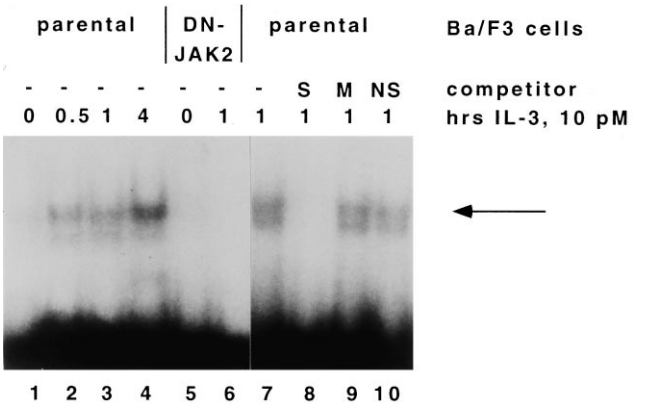

Fig. 4. IL-3-inducible protein binding to the Ets motif of the *DUB-1* enhancer element and suppression of binding in the presence of dominant-negative (DN) JAK2. Parental Ba/F3 cells and cells stably expressing DN-JAK2 were grown for 8 h in the absence of IL-3 and restimulated with the cytokine (10 pM) for the indicated periods of time. Gel mobility shift analysis was performed by incubating nuclear extracts with a ^{32}P-labeled probe derived from the IL-3 RE of *DUB-1* which contains the consensus sequence for Ets binding (probe 1 in Fig. 1). For competition analysis (lanes 8–10), a 100-fold molar excess of unlabeled specific probe (S), a mutant probe carrying the point mutation indicated in Fig. 1 in the Ets site (M) or a non-specific oligonucleotide (NS) was added to the binding reaction.

Oligonucleotide probes were generated from the *DUB-1* enhancer element that carried AP-1, GATA, CBF, or Ets motifs as single binding sites (Fig. 1).

As shown in Fig. 3 (left panel), IL-3 induced the binding of a protein complex to an oligonucleotide with the 5′ AP-1 site (probe 2 in Fig. 1). The protein binding was specific because it could be competed by an excess of unlabeled WT probe but not by a probe with a mutant AP-1 site or a random oligonucleotide (Fig. 3, middle panel). In order to identify proteins of the complex, we performed a supershift analysis. Because AP-1 complexes can be formed either by Fos-Jun hetero- or Jun homodimers [22], we used a set of different antibodies against these proteins. As Fig. 3 (right panel) indicates, the complex could be supershifted by a JunD antibody, but not by c-Jun, JunB, or pan-Fos antisera. Identical binding patterns were observed with the oligonucleotide (probe 5 in Fig. 1) carrying the 3′ AP site (data not shown). Therefore, our data identify JunD as a component of the protein complex binding to the AP-1 sites of the *DUB-1* IL-3 RE.

IL-3 also induced the binding of a protein complex to the oligonucleotide probe with the Ets motif (probe 1 in Fig. 1) of *DUB-1* (Fig. 4, lanes 1–4). An excess of unlabeled WT probe but not of a probe with a mutant Ets site (Fig. 1) or a random oligonucleotide had an inhibitory effect, indicating the specificity of protein binding (Fig. 4, lanes 8–10). So far, we could not identify the protein(s) binding to the Ets site of *DUB-1*. A supershift analysis using specific antibodies against several Ets family members suggested that the protein complex that binds to the Ets motif does not contain Ets-1, Ets-2, Elk-1, or SAP-1 (data not shown).

We have recently generated Ba/F3 subclones stably transfected with Δ829-JAK2, a dominant-negative deletion mutant of JAK2 [19], and shown that these cells do not express *DUB-1* mRNA or protein [15]. Interestingly, in cells stably expressing dominant-negative JAK2, there was also no detectable binding of

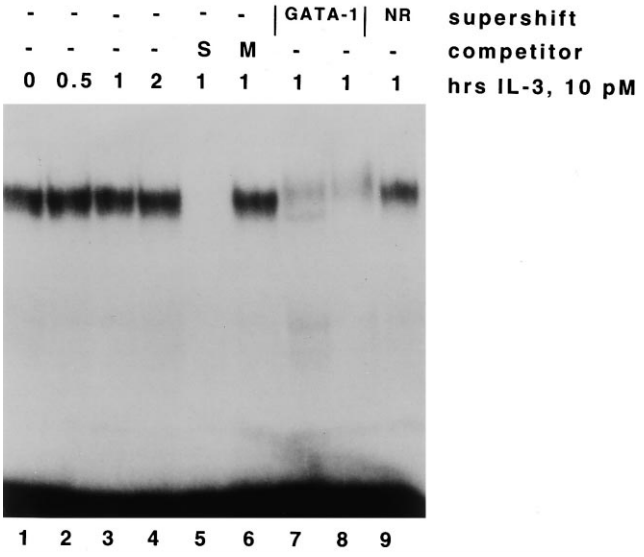

Fig. 5. Analysis of protein binding to the 5′ GATA motif of the *DUB-1* enhancer element. Ba/F3 cells were deprived of IL-3 for 8 h and restimulated with the cytokine (10 pM) for the indicated periods of time. Nuclear extracts of the cells were incubated with a ^{32}P-labeled oligonucleotide probe from the *DUB-1* enhancer element that contains the 5′ GATA site (probe 4 in Fig. 1). For competition analysis (lanes 5 and 6), a 100-fold molar excess of unlabeled specific probe (S) or a probe with a mutant GATA site (M) (Fig. 1) was included in the binding reaction. In lanes 7–9, antibodies to GATA-1 or, as a non-related control (NR), to STAT5 were incubated with the nuclear extracts as indicated. To test the effect of different reaction conditions, the antibody was added either after the binding reaction for an additional 20 min (lane 7), or 20 min prior to the addition of the labeled probe (lanes 8 and 9).

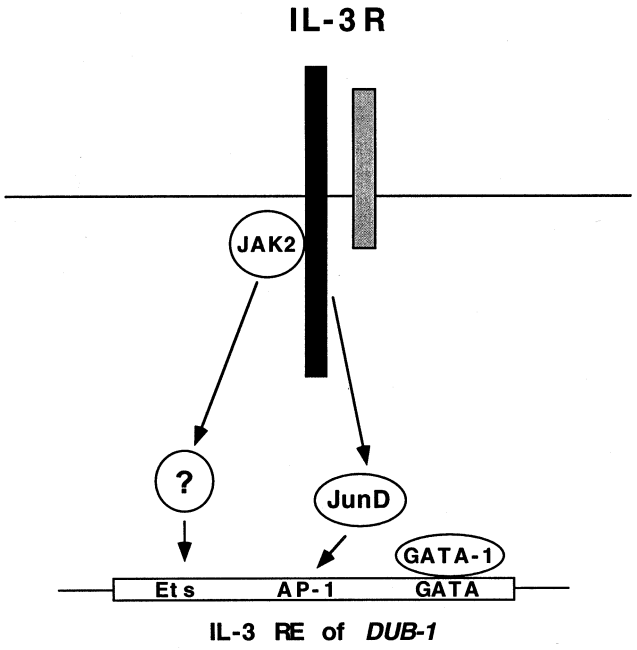

Fig. 6. A model for the activation of the IL-3 response element of *DUB-1*. An Ets site, a consensus sequence for GATA binding and two AP-1 sites (symbolized by one AP-1 motif) are required for *DUB-1* enhancer activity. In Ba/F3 cells, IL-3 induces the binding of JunD to the AP-1 sites and activates a novel signaling pathway, which requires JAK2 and an Ets site-binding protein. GATA-1 constitutively binds to the GATA motif of *DUB-1*.

the IL-3-inducible protein complex to the probe with the Ets site (Fig. 4, lanes 5 and 6). In contrast, the IL-3-dependent activation of AP-1 binding to the DUB-1 enhancer element was not affected by dominant-negative JAK2 (R.J., unpublished data), indicating that the observed inhibition of protein binding to the Ets motif does not reflect a non-specific suppression of cytokine-induced signaling events in the stably transfected cells. Furthermore, we have previously shown that two other cytokine-inducible genes, c-*fos* and c-*myc*, are normally expressed in response to IL-3 treatment [15].

To address the question of whether GATA proteins bind to the *DUB-1* enhancer element, we used an oligonucleotide (probe 4 in Fig. 1) carrying the 5′ GATA motif (Fig. 2). As shown in Fig. 5, a protein complex was detected that constitutively binds to this probe (lanes 1–4). The binding was suppressed in the presence of an excess of unlabeled WT probe (lane 5), indicating that binding was specific. An oligonucleotide with a mutant GATA site (Fig. 1) and a random oligonucleotide failed to inhibit protein binding to the labeled WT probe (lane 6 and data not shown). Furthermore, incubation of the nuclear extracts with an antibody to GATA-1 either prior to or after the addition of the ^{32}P-labeled probe strongly suppressed the probe-binding activity (lanes 7 and 8). The GATA-1 antibody had no effect on IL-3-stimulated protein binding to another oligonucleotide probe with a STAT5 consensus sequence [15], further excluding a non-specific inhibition (data not shown). Together, these data suggest that GATA-1 binds to the 5′ GATA motif of the *DUB-1* enhancer. No protein binding could be detected if an oligonucleotide containing a consensus sequence for CBF binding (probe 3 in Fig. 1) was used as a probe for gel mobility shift analysis (data not shown).

4. Discussion

Cytokine-inducible genes provide critical links between short-term signals generated at the cell membrane and long-term cellular responses. In this work, we have extended our studies on the regulation of the murine immediate-early gene *DUB-1* by IL-3. Continuous expression of the *DUB-1* gene product, a deubiquitinating enzyme, induces a growth arrest in the G_1 phase of the cell cycle [7], suggesting an involvement of the DUB-1 protein in the regulation of IL-3-stimulated cell growth, or cell cycle progression. Previous studies revealed an important role of JAK2 and the Ras-Raf-MAPKinase signaling cascade in *DUB-1* regulation [15], but how the activation of these pathways and *DUB-1* induction are coupled remained to be elucidated. Well documented substrates downstream from activated JAKinases are the members of the STAT family of transcription factors [4,23]. However, the minimal IL-3 RE of the *DUB-1* gene, a 112 base pair element located 1.4 kb upstream from the ATG start codon which retains the IL-3-inducible enhancer activity of the 5′ flanking region of *DUB-1* [8], lacks a consensus sequence for STAT binding. Our data indicate that two AP-1 and one GATA motif, in addition to a consensus sequence for Ets binding, are important *cis*-regulatory elements of the *DUB-1* gene. Previous studies have demonstrated the IL-3-inducible binding of protein complexes to probes derived from the IL-3

RE of *DUB-1* [8,15], but the probes contained consensus sequences for binding of two or more transcription factors, complicating the interpretation of the data. Here we show that IL-3 induces the binding of discrete protein complexes to both the AP-1 and the Ets sites of *DUB-1*.

We have identified JunD as a component of the AP-1 complex which binds to the IL-3 RE of *DUB-1*. In contrast to other Jun family members, such as c-Jun and JunB, which are important components in mitogenic signal transduction [24–26], JunD (if overexpressed) fails to stimulate cell growth [27], and some studies even suggested a negative growth-regulatory function [28,29]. The role of JunD in cytokine receptor signaling is not well established. In serum-stimulated fibroblasts, hyperphosphorylation of JunD, repression of JunD AP-1 DNA-binding activity and downregulation of JunD expression have been observed and may provide regulatory mechanisms of overriding the growth-suppressing effect of JunD [30]. Our results indicate that IL-3 can stimulate the DNA binding of JunD to the AP-1 sites of *DUB-1*, suggesting a role of this transcription factor in signal transduction from the IL-3 receptor. Further studies are necessary to investigate whether the DUB-1 protein might be involved in the mediation of the anti-proliferative effect of JunD.

The protein(s) binding to the Ets consensus sequence of *DUB-1* remain unidentified. Ets proteins are a large family of transcription factors [12], and their regulation as well as their specific target genes are intensively studied. While Ets proteins are required for the development of specific hematopoietic lineages [31–33], relatively little is known about their role in signal transduction downstream from cytokine receptors. The identification of an IL-3-inducible protein complex bound to the Ets site of *DUB-1* and the absence of this complex in Ba/F3 cells which do not express *DUB-1* suggest an important role of an Ets protein in the regulation of the *DUB-1* gene. The Ba/F3 subclones not expressing *DUB-1* have been generated by stable transfection with a dominant-negative deletion mutant of JAK2 [15]. Therefore, an attractive hypothesis is that JAK2 is upstream from an Ets protein in an IL-3-inducible signaling pathway involved in *DUB-1* induction (Fig. 6).

Remarkably, a mutation in the Ets motif of DUB-1 not only reduced the IL-3-inducible enhancer activity but also the constitutive activity (Fig. 2). Using a probe derived from the DUB-1 enhancer that contained the Ets site together with the neighboring 5′ AP site we have previously shown that both constitutive and IL-3-inducible protein complexes bind to this region of the IL-3 RE [8]. In contrast, a probe with the isolated Ets motif binds only the inducible complex (Fig. 4). Therefore, it is conceivable that the constitutive binding of an additional protein complex contributes to the basal enhancer activity, and that this binding requires both an intact Ets and AP-1 site, analogous to the binding of, for example, NFAT to the IL-2 enhancer [34].

Additional gel-shift experiments revealed that the GATA-1 protein constitutively binds to the *DUB-1* enhancer element. Together with transcriptional reporter assays, indicating that this GATA site is required for *DUB-1* enhancer activity, this observation suggests an important role of GATA-1 in the regulation of *DUB-1* expression. GATA proteins are key regulators of mammalian hematopoiesis [13,35]. Expression of the transcription factor GATA-1 is almost entirely restricted to hematopoietic tissues (erythroid, mast, megakaryocytic, and multipotential progenitor cells), with Sertoli cells of the testis as the only known exception [13,35]. GATA-1-binding to the *DUB-1* enhancer element, therefore, could be important for the hematopoietic-specific expression pattern of *DUB-1*. Interestingly, the GATA site, in contrast to the Ets and AP-1 motifs, is not conserved in another *DUB* gene family member, *DUB-2A* (K.-H.B., manuscript in preparation), further suggesting a role of this site in directing tissue-specific expression.

DUB proteins are a new family of deubiquitinating enzymes which are expressed in response to hematopoietic cytokines [7,8,36]. Protein ubiquitination and deubiquitination represent central mechanisms for the control of protein stability. Polyubiquitinated proteins are rapidly degraded by the 26S proteasome, a multisubunit protein degradation complex [37–39]. In addition, recent evidence suggests that ubiquitination may be a reversible post-translational modification, regulating cellular targeting and enzymatic activity [37]. The biological processes controlled by protein ubiquitination are diverse and include, for example, cell cycle progression, apoptosis, processing

of antigens, the heat-shock response, and modulation of important cell surface receptors and transcriptional regulators [37–39].

The cellular targets of DUB proteins remain unknown. Further studies on their function and cytokine-dependent regulation may contribute to the understanding of the specificity of cytokine action and the relationships between cytokine receptor signaling and the regulation of protein degradation.

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