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IL-3 dependent regulation of Bcl-x_L gene expression by STAT5 in a bone marrow derived cell line

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Activation of the Jak/STAT pathway by cytokines has been shown to regulate differentiation, proliferation or apoptosis in hematopoeitic cells. Among the Stat proteins, STAT5 is activated by a broad range of cytokines. In order to study the role of STAT5 in hematopoietic cells, we stably expressed a dominant negative form of STAT5 (STAT5AΔ749) in the IL-3 dependent bone marrow derived Ba/F3 cell line. Ba/F3 cells expressing STAT5AΔ749 were found to be more sensitive to apoptosis than parental or control Ba/F3 cells after IL-3 withdrawal. Analysis of the expression of the cell death regulators, Bcl-2 and Bcl-x, revealed that the level of Bcl-x was lower in Ba/F3 cells expressing STAT5AΔ749 than in control cells. IL-3 regulation of Bcl-x expression at protein and mRNA levels was impaired in these cells while that of Bcl-2 expression was unaffected. We further demonstrated that the Bcl-x gene promoter contained a proximal STAT consensus sequence that bound STAT5. Transactivation of a Bcl-x gene promoter reporter construct by STAT5 was observed in Ba/F3 cells. Introduction of a mutation in the STAT binding site abolished this transactivation. These data indicate that Bcl-x is probably a STAT5 target gene. They also support the involvement of STAT5 in hematopoietic cell survival.

Keywords: apoptosis; STAT5; Bcl-x_L; transcription

Introduction

Hematopoietic growth factors maintain hematopoietic homeostatis by regulating cellular proliferation, differentiation or survival. Binding of these polypeptide ligands to cell surface receptors triggers distinct signaling pathways. One of them involves the activation of STAT proteins (Signal Transducer and Activator of transcription) by cytokine receptor-associated tyrosine kinases of the Jak family (Darnell *et al*., 1994; Ihle, 1996). The STAT proteins are latent cytoplasmic transcription factors that become tyrosine phosphorylated after ligand binding (Shuai *et al*., 1993; Gouilleux *et al*., 1994). Activated STATs dimerize (homo- or hetero-dimerize), migrate into the nucleus, bind to specific DNA elements and activate the transcription of responsive genes. All STAT members contain in their structure a SH2 domain which allows their selective recruitment to the receptor-kinase activated complex and the dimerization of the STATs (Heim *et al*., 1995; Shuai *et al*., 1994; Stahl *et al*., 1995). Functional domains involved in DNA binding and activation of transcription have been defined (Horvath *et al*., 1995; Schindler *et al*., 1995; Moriggl *et al*., 1996, 1997; Schaefer *et al*., 1997).

Seven mammalian STAT proteins that display a highly specific role in innate and acquired immunity have been isolated: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. In addition, isoforms of STAT1, STAT3, STAT5A, STAT5B that lack the COOH terminus, a region highly variable among STAT members containing the transactivation domain, have been described. These isoforms arise through an alternative splicing mechanism or a specific protein processing and are thought to be natural dominant negative forms of STAT proteins (Schindler *et al*., 1992; Azam *et al*., 1995, 1997; Caldenhoven *et al*., 1996; Kazansky *et al*., 1995; Meyer *et al*., 1998; Schaefer *et al*., 1997; Wang *et al*., 1996).

STAT5 has been originally identified in the mammary gland as a prolactin inducible STAT protein that regulated milk protein gene expression (Wakao *et al*., 1994). Two highly related STAT5 molecules, STAT5A and STAT5B, encoded by distinct genes have been isolated (Liu *et al*., 1995; Mui *et al*., 1995). STAT5A−/− mice have a defect in mammary gland development and lactation (Liu *et al*., 1997). Additional phenotypes associated with the loss of growth hormone responses were observed in STAT5B−/− (Udy *et al*., 1997). STAT5 is activated by a wide range of cytokines including IL-2, IL-3, GM-CSF, erythropoietin (EPO), thrombopoietin (TPO), growth hormone or by oncogenes like v-*abl*, bcr-abl and v-*mpl* (Gouilleux *et al*., 1995; Pallard *et al*., 1995a, b; Wakao *et al*., 1995; Chai *et al*., 1997; Danial *et al*., 1995; Wood *et al*., 1995). Constitutive activation of STAT5 in blood cells from leukemic patients has also been reported (Gouilleux-Gruart *et al*., 1996). Conflicting results were obtained on the role of STAT5 in the proliferation of hematopoietic cell lines. The use of cytokine receptor mutants unable to activate STAT5, or the use of dominant negative form of STAT5, indicated that STAT5 activation was required for Epo, IL-2- or IL-3-induced cell proliferation, while other studies suggested that STAT5 was not essential for this effect (Friedmann *et al*., 1996; Fujii *et al*., 1995; Gobert *et al*., 1996; Mui *et al*., 1996; Quelle *et al*., 1996). Studies with dominant negative STAT5 and receptor

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mutants also demonstrated a role of STAT5 in erythroid differentiation (Chretien *et al*., 1996; Iwatsuki *et al*., 1997). More recently, it has been shown that STAT5 might be involved in the regulation of apoptosis in hematopoietic cells (Rui *et al*., 1998; Zamorano *et al*., 1998). In STAT5A−/− mice, proliferation of macrophages in response to GM-CSF was shown to be affected, and in mice where STAT5A and STAT5B genes were simultaneously inactivated, consistent reduction in the size and in the number of hematopoietic progenitor colonies induced by IL-3, GM-CSF and IL-5 were observed (Feldman *et al*., 1997; Teglund *et al*., 1998). Despite these disparate biological effects, a few STAT5 regulated genes have been identified in hematopoietic cells: CD25, oncostatin (OSM) and CIS genes that contain in their promoter region one or several STAT5 binding sites (Lecine *et al*., 1996; Matsumoto *et al*., 1997; Yoshimura *et al*., 1996; Verdier *et al*., 1998).

In order to identify new STAT5 target genes, we established cell lines that express a dominant negative form of STAT5 (STAT5AΔ749) in the IL-3 dependent hematopoietic Ba/F3 cells. Cells expressing STAT5AΔ749 are more sensitive to apoptosis than parental or control Ba/F3 cells in absence of IL-3. We showed that STAT5AΔ749 expression inhibits Bcl-x_L protein and mRNA expression induced by IL-3. We further demonstrated by EMSA that the Bcl-x gene promoter contains a proximal response element that binds STAT5. Co-transfection experiments with STAT5A and STAT5B expression vectors and the Bcl-x promoter luciferase construct indicated that STAT5 regulates transcription of the Bcl-x gene. The identification of an anti-apoptotic gene as a target gene of STAT5 supports the role of this transcription factor in the suppression of apoptosis elicited by cytokines.

Results

Expression and activation of STAT5AΔ749 in Ba/F3 cells

In order to study the role of STAT5 in hematopoietic cells, we stably expressed a truncated version of STAT5, STAT5AΔ749 in the bone marrow derived cell line, Ba/F3. This dominant negative mutant contains a deletion of the last 45 amino acids that removes the transactivation domain but retains its DNA binding capacity (Moriggl *et al*., 1996). Ba/F3 cells were transfected with a pRSV/neo vector or a pRSVneo vector containing STAT5AΔ749, cDNA and stable G418 resistant cells were obtained. We first determined expression and activation of STAT5AΔ749 in two independent cell clones (Figure 1). Cell extracts from Ba/F3neo and Ba/F3Δ749 cells were analysed in Western blot with an antibody raised against the SH2–SH3 region of STAT5 that recognized STAT5A, STAT5B and STAT5AΔ749. A 97 kDa band corresponding to the known molecular weight of STAT5A and STAT5B was detected in the cell extracts of Ba/F3neo control cells (Figure 1a, lanes 1 and 2), whereas an additional 84 kDa band corresponding to the molecular weight of STAT5AΔ749, was observed in cell extracts of Ba/F3Δ749 cell clones 31 and 32 (lanes 3 and 4). In clone 31, levels of the endogenous expression of STAT5A and STAT5B and of transfected STAT5AΔ749 expression were similar. In clone 32, expression of STAT5AΔ749 was lower than that of the full-length STAT5. Whole cell extracts from IL-3 stimulated or non stimulated transfected cells (Ba/F3Δ749, clone 31) or from control cells (Ba/F3neo) were analysed in a band shift experiment with the specific STAT5 binding sequence from the β-casein gene promoter as probe. In Ba/F3neo and in Ba/F3Δ749 cells, IL-3 induced the appearance of complexes with distinct electrophoretic mobilities (Figure 1b, lanes 2 and 7). To identify these complexes, we used two specific antibodies raised against the COOH terminal region of STAT5A and STAT5B unable to recognize STAT5AΔ749, and one antibody raised against the NH2-terminal region of STAT5A that recognizes the three STAT5 forms. These antibodies were incubated with whole cell extracts from stimulated cells, and the appearance of a supershift was monitored. In Ba/F3neo cells, the IL-3 inducible complex was almost supershifted by the STAT5A and the STAT5 NH2-terminal antibodies and weakly by the STAT5B antibody (Figure 1b, lanes 3–5). In Ba/F3Δ749 cells, this complex was only supershifted by the STAT5 NH2-terminal antibody (Figure 1b, lanes 8–10). No supershift was detected with the anti-STAT5A and anti-STAT5B antibodies although heterodimerization of STAT5Δ749 with STAT5A and STAT5B was observed in co-immuno-

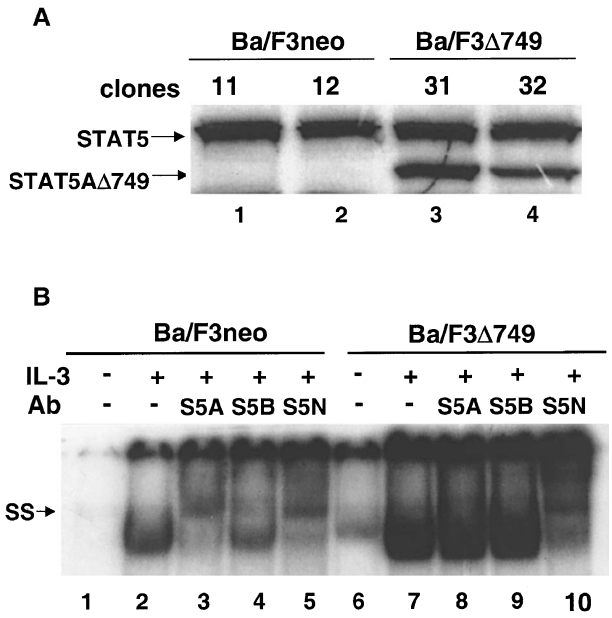

Figure 1 Expression and activation of STAT5AΔ749 in Ba/F3 cells. (**a**) Western blot analysis of Ba/F3neo and Ba/F3Δ749 cell clones. Ba/F3 cells were transfected with a pRSVneo control plasmid (lanes 1 and 2) or the pRSV STAT5AΔ749neo plasmid (lanes 3 and 4). Western blot was performed with a rabbit polyclonal STAT5 antibody. The positions of STAT5A/B and the truncated STAT5AΔ749 are indicated. (**b**) Nuclear extracts from unstimulated Ba/F3neo (lane 1), Ba/F3Δ749 cells (lane 6) IL-3 stimulated Ba/F3neo (lanes 2–5) or Ba/F3Δ749 cells (lanes 7–10) were prepared and analysed in a band shift experiment with a β-casein oligonucleotide as probe. Nuclear extracts were incubated with antibodies raised against the COOH terminal end of STAT5A (S5A) (lanes 3 and 8), the COOH terminal end of STAT5B (S5B) (lanes 4 and 9) or the NH2 terminal region of STAT5A and B (S5N) (lanes 5 and 10). Supershifts are indicated (SS)

precipitation experiments (data not shown). We did not detect STAT1 or STAT3 activation in Ba/F3Δ749 cells or Ba/F3neo cells after IL-3 stimulation, even with oligonucleotide probes that interact specifically with STAT1 and STAT3 (data not shown). Thus, the complex induced by IL-3 in Ba/F3Δ749 cells (clone 31) contained mainly the STAT5AΔ749 form. Similar results were obtained with cells from clone 32.

Dominant negative effect of STAT5AΔ749

STAT5AΔ749 has been shown to inhibit the transactivation of the β-casein gene promoter by STAT5A or STAT5B in transfected COS cells (Moriggl *et al*., 1996). We examined the dominant negative effect of STAT5AΔ749 on the endogenous STAT5 activity in Ba/F3 cells. Ba/F3neo or Ba/F3Δ749 cells were transfected with a TK luciferase or a six times copy of the STAT5 binding site in front of the TK luciferase reporter gene construct. Luciferase activities were determined in absence or presence of IL-3 (Figure 2). Transfection of the (STAT5)x6TK luciferase construct showed a 3–4-fold induction of the luciferase activity by IL-3 in Ba/F3neo cells but not in Ba/F3Δ749 cells, while the luciferase basal and induced activities of the TK promoter were similar in both cell lines (Figure 2). Thus, expression of STAT5AΔ749 in Ba/F3 cells inhibited the transactivation of a STAT5 regulated promoter.

Apoptosis evaluation parental Ba/F3, Ba/F3neo and Ba/F3Δ749 cells after IL-3 withdrawal

The role of STAT proteins in the regulation of apoptosis has been recently described (Fukada *et al*., 1996; Rui *et al*., 1998; Zamorano *et al*., 1998; Schindler, 1998). We analysed the contribution of STAT5 in the suppression of apoptosis in Ba/F3 in absence or presence of IL-3. Apoptosis of parental Ba/F3, Ba/F3neo and Ba/F3Δ749 cells after IL-3 withdrawal was determined by agarose gel electrophoresis to detect the characteristic DNA fragmentation (Figure 3a). After 24 h of IL-3 starvation, DNA fragmentation was observed in Ba/F3Δ749 cells but not in parental and control Ba/F3 cells. Viability was also measured by propidium iodide exclusion (Figure 3b). Almost 20% of living cells were present in culture when Ba/F3Δ749 cells were deprived of IL-3 for 24 h, while 80% of cells were still viable in parental and control Ba/F3 cell cultures. In presence of IL-3, we failed to detect an apoptosis in Ba/F3 cells expressing STAT5AΔ749, despite the persistent presence of a higher percentage of dead cells (data not shown). These data indicated that expression of STAT5AΔ749 increased the susceptibility of Ba/F3 cells to undergo apoptosis after IL-3 withdrawal.

Expression and regulation of Bcl-2 member proteins in Ba/F3Δ749 cells

One of the key events that promotes suppression of apoptosis by cytokines is the regulation of the cell death regulators such as the Bcl-2 family members (Park, 1996). Relative levels of Bcl-2 and Bcl-x were measured by immunoblot at various times after depriving the different Ba/F3 cell lines of IL-3 (Figure 4a). The level of Bcl-x was lower in Ba/F3Δ749 cells than that of Ba/F3neo or parental Ba/F3 cells at every time point of starvation. In Ba/F3Δ749 cells, level of Bcl-x was dramatically reduced and barely detectable after 24 h of IL-3 starvation (Figure 4a and b). Levels of Bcl-2 were similar in parental Ba/F3 cells and in Ba/F3Δ749 cells, and higher in Ba/F3neo cells. After 24 h

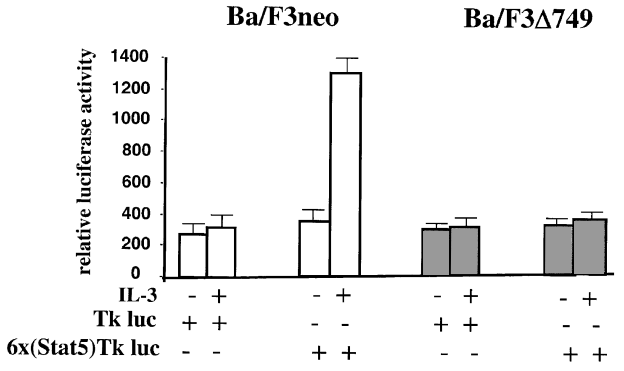

Figure 2 Dominant negative effect of STAT5AΔ749 in stably transfected Ba/F3 cells. Ba/F3neo or Ba/F3Δ749 cells were transfected with a TK-luciferase or a (STAT5)x6TK luciferase constructs. Cell extracts were prepared and luciferase activities were determined in absence or presence of IL-3. Results are the mean of three independent experiments

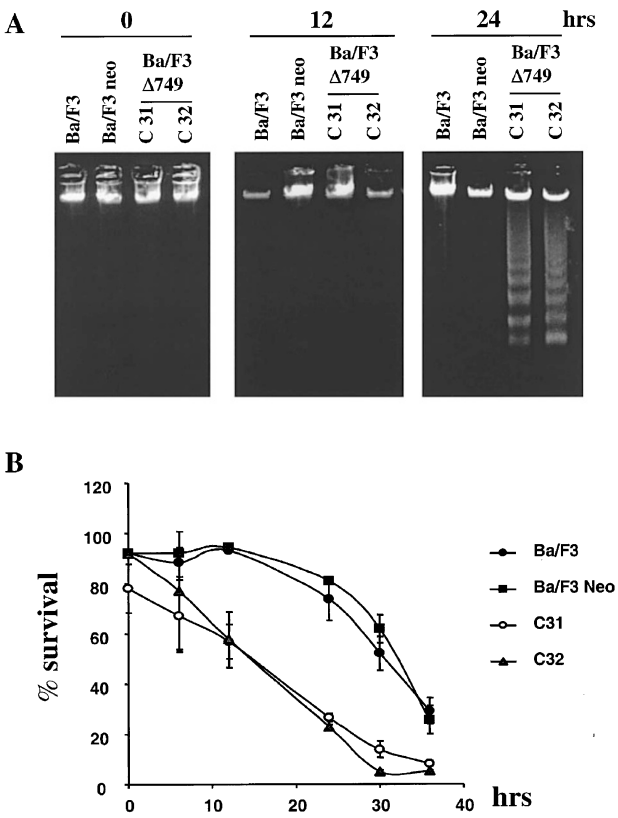

Figure 3 Apoptosis evaluation in Ba/F3Δ749 cells. (**a**) After washing, parental Ba/F3, Ba/F3neo and Ba/F3Δ749 cells were plated without Il-3 and harvested at the indicated hours. DNA was extracted from the cells and run on agarose gels to look for the presence of the DNA fragmentation characteristic of apoptosis. (**b**) At the same time, cell viability was determined by propidium iodide exclusion. Results are presented as mean viability of three independent experiments

of IL-3 starvation, the level of Bcl-2 decreased in Ba/F3Δ749 cells. We next analysed the importance of STAT5AΔ749 on the regulation of Bcl-2 and Bcl-x expressions by IL-3 in Ba/F3 cells. Cell extracts from Ba/F3Δ749 (clone 31) or Ba/F3neo cells stimulated for various lengths of time with IL-3 were prepared and analysed by Western blot with anti-Bcl-2 or anti-Bcl-x antibodies (Figure 5). IL-3 induced similar levels of Bcl-2 expression in Ba/F3neo and in Ba/F3Δ749 cells (upper panel). In contrast, clear differences in the regulation of Bcl-x expression were observed between Ba/F3Δ749 and Ba/F3neo cells. Bcl-x expression was induced in Ba/F3neo cells after 2 h stimulation by IL-3 and expression increased up to 8 h. In Ba/F3Δ749 cells, regulation of Bcl-x expression by IL-3 was dramatically affected and only a slight induction was observed after 2 h stimulation. Similar results were also obtained with clone 32 (data not shown). Bax expression was also analysed in both cell lines. Bax was detected in absence of IL-3 but was not induced by IL-3 in Ba/F3neo and in Ba/F3Δ749 cells (lower panel). We concluded that expression of STAT5AΔ749 impaired the regulation of Bcl-x expression by IL-3 in Ba/F3 cells.

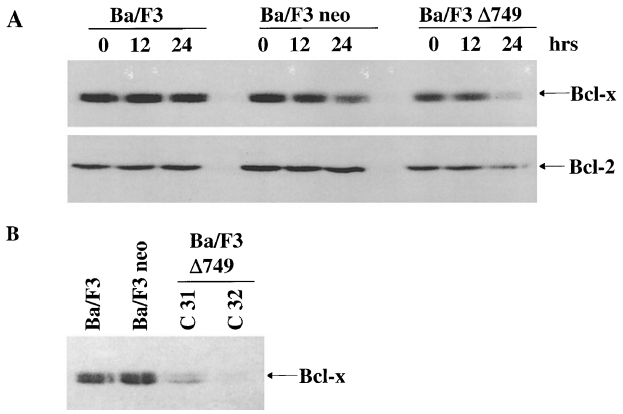

Figure 4 Expression of Bcl-2 and Bcl-x in parental Ba/F3, Ba/F3neo and Ba/F3Δ749 cells after IL-3 withdrawal. (**a**) After washing, Ba/F3 cells were plated without IL-3 and harvested at the indicated hours to determine the levels of Bcl-2 and Bcl-x protein by Immunoblot. (**b**) Bcl-x expression in Ba/F3Δ749 cells (clones 31 and 32) after 24 h of IL-3 starvation

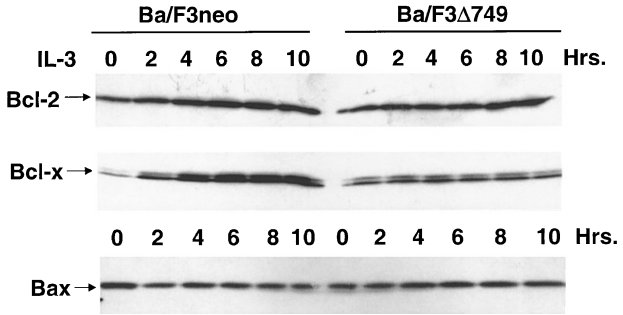

Figure 5 Regulation of Bcl-2, Bcl-x and Bax protein expression in Ba/F3neo and Ba/F3Δ749 cells. Ba/F3neo and Ba/F3Δ749 cells (clone 31) were treated with IL-3 for different lengths of time as indicated. Cell extracts were prepared and analysed by Western blot with anti-Bcl-2 (upper panel), anti-Bcl-x (middle panel) and anti-Bax (lower panel) antibodies

Regulation of Bcl-x_L mRNA expression by IL-3 in Ba/F3neo and in Ba/F3Δ749 cells

We next compared the induction of Bcl-x mRNA in Ba/F3neo and in Ba/F3Δ749 cells by IL-3 by Northern blot analysis (Figure 6a). RNAs were prepared from both cell lines treated for various lengths of time with IL-3. Bcl-x mRNA expression was induced after 2 h stimulation with IL-3 in Ba/F3neo cells and reached a maximum at 3.5 h. Similar observations were made in Ba/F3Δ749 cells. However, the level of expression was strongly reduced in these cells. As a control, the RNA blot was reprobed with a cDNA encoding the murine CIS gene, a well known target gene of STAT5 (Figure 6a). CIS mRNA expression was induced after 30 min stimulation with IL-3 in Ba/F3neo cells with a peak of expression at 2 h. This induction was also observed in Ba/F3Δ749 cells, but the level of CIS mRNA expression was reduced. As a control for equivalent RNA loading, the membrane was further reprobed with a GAPDH cDNA. Bcl-x, CIS and GAPDH mRNAs were next quantified and the ratios Bcl-x/GAPDH, CIS/GAPDH were plotted for both cell types (Figure 6b). At the maximum of IL-3 stimulation, expression of Bcl-x and CIS mRNAs in Ba/F3Δ749 cells were 3–4-fold lower than in Ba/F3neo cells. These data indicated that STAT5AΔ749 expression interfered with the IL-3 regulation of Bcl-x and CIS mRNA expression in Ba/F3 cells.

In humans and rodents, two different Bcl-x mRNA species that differ by a region of 189 bp located in the 3′ region of exon II, have been identified. To discriminate between the long form (Bcl-x_L, anti-apoptotic) and the short form (Bcl-xS, pro-apoptotic), we performed RT–PCR reactions with a couple of specific primers that allows identification of the Bcl-x mRNA species. PCR products of 502 bp for the long form and 312 bp for the short form were expected. RNAs from Ba/F3neo and Ba/F3Δ749 cells treated for different times with IL-3 were used in the RT–PCR reactions (Figure 7). Untreated cells or cells treated with IL-3 expressed the amplified coding region of Bcl-x_L. We did not detect the presence of the Bcl-xS transcript in either cell lines. As observed in Northern blot, levels of the PCR products in Ba/F3Δ749 cells were weakly induced by IL-3 compared to that of Ba/F3neo cells. Identity of the PCR product was further confirmed by Southern blot with a Bcl-x probe (middle panel). RT–PCR reactions with actin primers were also done as control. Thus, STAT5AΔ749 inhibited the regulation of Bcl-x_L mRNA expression in Ba/F3 cells.

STAT5 binds to a proximal element in the Bcl-x gene promoter

Sequence alignment of the human and mouse Bcl-x gene promoters revealed the presence of a STAT binding motif at position −332 in the murine sequence and at position −323 in the human sequence upstream the start codon (Grillot *et al*., 1997). We analysed the capacity of STAT5 to bind to this proximal STAT binding site and to transactivate the Bcl-x gene promoter. Nuclear extracts from IL-3 stimulated Ba/F3neo and Ba/F3Δ749 cells were prepared and analysed in a band shift experiment with an oligonucleotide from −336 to −316 that contained

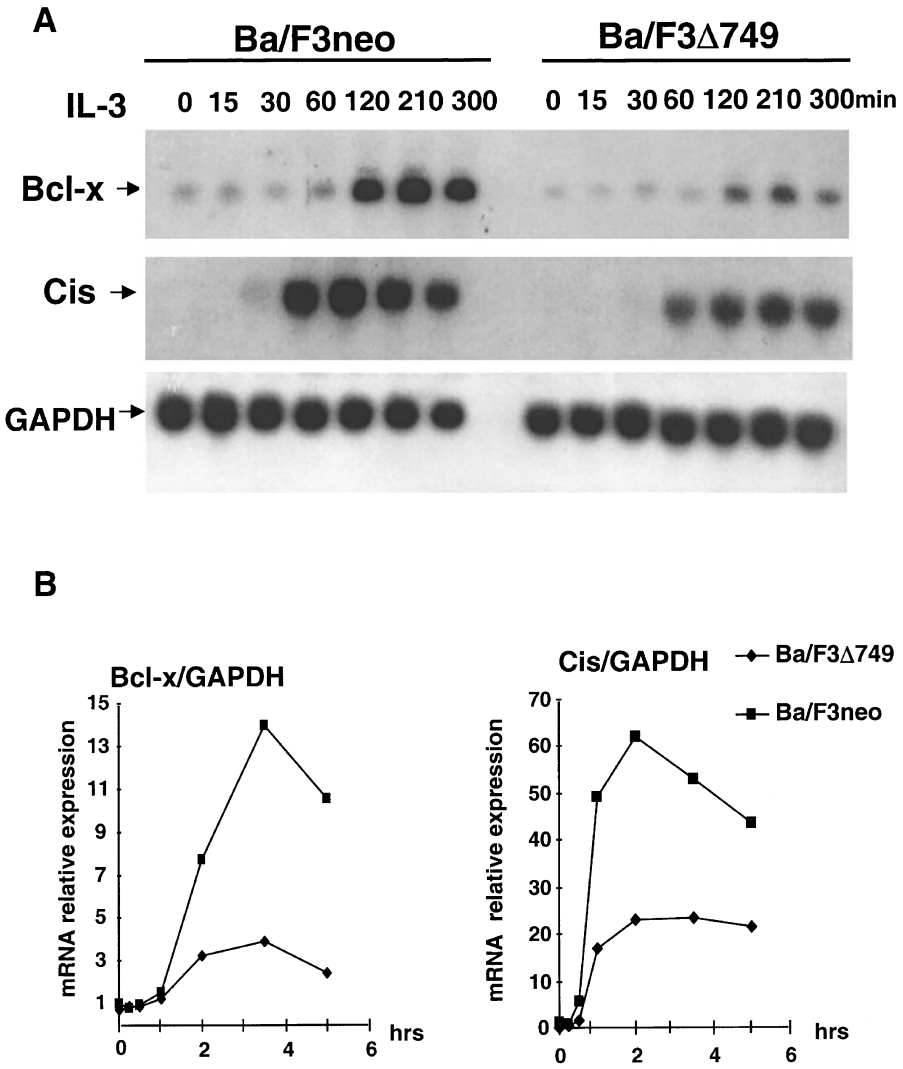

Figure 6 (**a**) Regulation of Bcl-x mRNA expression in Ba/F3neo and Ba/F3Δ749 cells. RNAs were prepared from Ba/F3neo and Ba/F3Δ749 cells (clone 31) treated with IL-3 for 0 to 300 min as indicated. Northern blots were hybridized with a Bcl-x probe (upper panel). Membranes were subsequently tested with a CIS probe (middle panel) and a GAPDH probe as control (lower panel). (**b**) Quantification of Bcl-x mRNA in Ba/F3neo and Ba/F3Δ749 cells. Bcl-x and CIS mRNA levels were normalized to levels of GAPDH mRNA in both cell lines and plotted as a function of time of IL-3 exposure

the STAT binding site as probe (Figure 8a). IL-3 induced the formation of a DNA binding complex in Ba/F3neo and Ba/F3Δ749 cells with different mobilities (Figure 8, lanes 2 and 5). Nuclear extracts were incubated with the STAT5 antibody raised against the NH2-terminal part of STAT5, and the appearance of a supershift was monitored. Both complexes were supershifted by the STAT5 antibody (Figure 8, lanes 3 and 6). These data demonstrated that STAT5A and/or STAT5B and STAT5AΔ749 bound to the STAT binding site of the Bcl-x gene promoter.

Transcriptional regulation of the Bcl-x gene promoter by STAT5

The Bcl-x gene promoter was isolated from human genomic DNA by PCR and cloned upstream of the luciferase gene reporter. This construct was transfected in parental Ba/F3 cells with expression vectors for STAT5A and STAT5B or the empty vector. Cell extracts were prepared from IL-3 stimulated cells and the luciferase activities were determined (Figure 8b). A threefold induction of the luciferase activity was observed when STAT5 expression vectors were co-transfected (2.65 ± 0.5). Point mutation was introduced in the STAT5 binding site of the Bcl-x gene promoter. A C mutation was replaced by a T, a mutation that has already been shown to abolish DNA binding of STAT5 to the β-casein sequence (Gouilleux *et al*., 1995). The mutated Bcl-x promoter luciferase construct was transfected in Ba/F3 cell in the presence or absence of the STAT5A and STAT5B expression vectors. Transactivation by STAT5 was not observed in this case. These results indicated that STAT5 was involved in the transcriptional regulation of the Bcl-x gene promoter.

Discussion

STAT5 is activated by a wide variety of cytokines that promote differentiation, proliferation or suppression of

apoptosis in hematopoietic cells. Previous studies indicated that STAT5 might be important for these three biological effects. However, the STAT5 target genes that could be involved in these distinct processes remain poorly known. We sought to identify genes that could be regulated by STAT5 by expressing a dominant negative form of STAT5 (STAT5AΔ749) in the IL-3 dependent cell line Ba/F3. The dominant negative effect has been determined by two ways: STAT5AΔ749 blocked the transactivation of an artificial STAT5 regulated promoter and inhibited the IL-3 dependent regulation of endogenous CIS gene expression.

We showed that expression of this dominant negative mutant increases the susceptibility of Ba/F3 cells to undergo apoptosis after IL-3 withdrawal but fails to inhibit the suppression of apoptosis induced by IL-3 in Ba/F3 cells, suggesting that other independent pathways activated by IL-3 contribute to the inhibition of apoptosis in Ba/F3 cells. IL-3 activates the PI-3 kinase-AKT pathway which has been shown to play a key role in cell survival (Franke *et al*., 1997). Active AKT phosphorylates the pro-apoptotic protein Bad, which dissociates from Bcl-x and this promotes the Bcl-x anti-apoptotic activity (del Peso *et al*., 1997). In addition, the activation of the PI-3 kinase-AKT pathway regulates the expression of Bcl-x (Leverrier *et al*., 1999). Regulation of Bcl-x gene expression and of Bcl-2 phosphorylation via the Ras/MAP kinase pathway and a PKC dependent pathway have also been reported (Leverrier *et al*., 1997; Kinoshita *et al*., 1995b; Ito *et al*., 1997). Thus, the inhibition of STAT5 activity may not be sufficient to induce apoptosis in presence of IL-3 in Ba/F3 cells.

The role of STAT proteins in the regulation of apoptosis in response to distinct cytokines has been also described. Studies on the tyrosine residues of the GP130, a common transducer chain of the IL-6 receptor, identified STAT3 as an important anti-apoptotic molecule, and STAT1 promotes cardiac myocytes survival in response to LIF (Fukada *et al*., 1996; Fujio *et al*., 1997). Cells expressing an IL-2 receptor β chain mutant unable to activate STAT5 were not protected from apoptosis by IL-2 and consequently could not be grown for a long-term in presence of IL-2 (Zamorano *et al*., 1998). STAT5 activation has been also observed in a T cell line upon treatment with an anti-apoptotic reagent or in Ba/F3 cells transformed by the bcr-abl oncogene that promotes a strong anti-apoptotic effect (Rui *et al*., 1998; Ahmed *et al*., 1998) indicating that STAT5 in these different situations might be an inhibitor of apoptosis. The reduction of Bcl-x expression but not that of Bcl-2 precedes the onset of apoptosis in T cells after IL-2 withdrawal and in Ba/F3 cells after IL-3 withdrawal and is thought to be a major pleiotropic anti-apoptotic gene in Ba/F3 cells (Broome *et al*., 1995; Leverrier *et al*., 1997; Thomas *et al*., 1998). This is in agreement with our results showing the dramatic reduction of Bcl-x expression in Ba/F3Δ749 cells in absence of IL-3. Similarly, it has been shown that suppression of apoptosis mediated by Bcr-abl or v-*abl* was independent of Bcl-2 expression but was correlated with up-regulation of Bcl-x_L (Amarente-Mendes *et al*., 1998; Chen *et al*., 1997). Altogether these results support our data that inhibition of Bcl-x by the dominant negative form of STAT5 coincide with an increase of apoptosis in Ba/F3 cells in absence of IL-3. However, from our data, the reduction of Bcl-2 levels

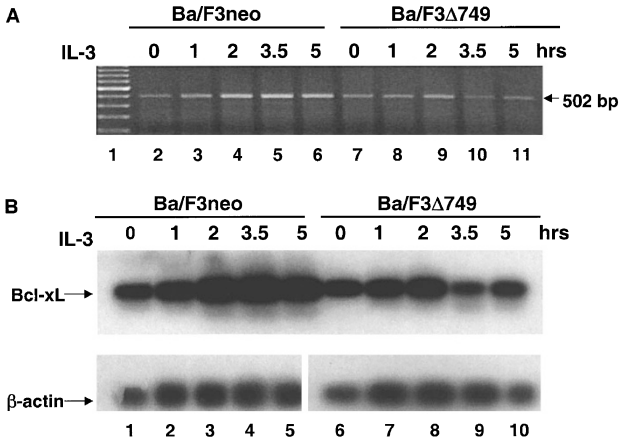

Figure 7 Induction of Bcl-x_L in Ba/F3neo and Ba/F3Δ749 cells. (**a**) RT–PCR reactions were performed with RNA from Ba/F3neo (lanes 2–6) and Ba/F3Δ749 cells (lanes 7–11) treated with IL-3 for 1 to 5 h. Molecular weight markers are loaded on lane 1 (100 bp ladder). (**b**) RT–PCR reactions were also carried on with actin primers as control. Identity of the PCR products were confirmed by Southern blot with a Bcl-x probe (upper panel) and an actin probe (lower panel)

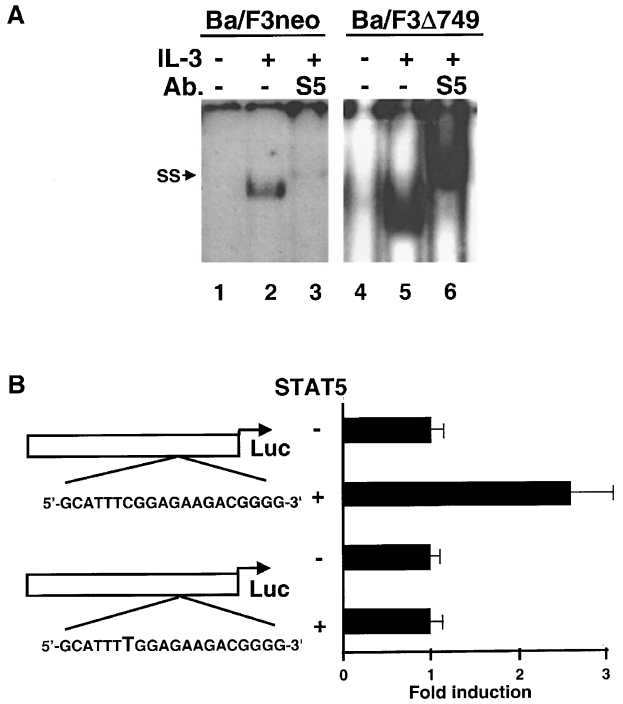

Figure 8 (**a**) DNA binding activity of STAT5 to the Bcl-x element (−336; −316). Nuclear extracts from Ba/F3neo and Ba/F3Δ749 cells were untreated (lanes 1 and 4) or treated with IL-3 for 30 min (lanes 2–3; 5–6) and analysed in a band shift experiment with a Bcl-x oligonucleotide as probe. Nuclear extracts were incubated with an anti-NH2-terminal STAT5 antibody (S5) and supershifts were monitored (lanes 3 and 6). Supershift is indicated (SS). (**b**) Transactivation of Bcl-x gene promoter by STAT5. The Bcl-x promoter luciferase construct and its mutated version were transfected in parental Ba/F3 cells together with a control vector or with STAT5A and STAT5B expression vectors (STAT5). Cell extracts were prepared and the luciferase activities were determined. Results are the mean of three independent experiments

observed after 24 h of IL-3 deprivation in Ba/F3Δ749 cells would suggest that Bcl-2 might contribute also to the apoptosis observed in these cells. Cytokines suppress apoptosis by regulating expression or phosphorylation of the Bcl-2 gene family members (Park, 1996; Broome *et al*., 1995; del Peso *et al*., 1997). In the present study, we provided evidence that STAT5 regulates the expression of the anti-apoptotic form of Bcl-x (Bcl-x_L) but not the expressions of Bcl-2 or Bax in Ba/F3 cells in response to IL-3. Moreover, induction of Bcl-2 expression by IL-3 was observed in Ba/F3Δ749 cells, indicating that STAT5 is not required for the regulation of Bcl-2 expression. Truncated IL-2 receptor β-chain, unable to activate STAT5, supported the Bcl-2 protein levels as the wild type receptor, indicating that regulation of Bcl-2 by IL-2 is also independent of STAT5 activation (Zamorano *et al*., 1998). However, from our experiments, we cannot exclude that STAT5 regulates the phosphorylation of Bcl-2. Indeed, phosphorylation of Bcl-2 after IL-3 stimulation has been shown to contribute to the inhibition of apoptosis (Ito *et al*., 1997).

Expression of STAT5AΔ749 in Ba/F3 cells inhibits the expression of Bcl-x_L at the level of protein and mRNA. Sequence comparison of human and murine Bcl-x gene promoters showed the presence of a STAT binding site (Grillot *et al*., 1997). Binding of STAT1 after activation by LIF and transactivation of the Bcl-x promoter by STAT1 was observed in cardiac myocytes (Fujio *et al*., 1997). We demonstrate here that this STAT element binds also STAT5 after activation by IL-3 in Ba/F3 cells, and that binding of STAT5 is required for the transcriptional activity of the Bcl-x gene promoter induced by STAT5 in presence of IL-3. In Ba/F3Δ749 cells, Bcl-x mRNA expression was still regulated by IL-3, indicating that other factors or signaling pathways are required for the IL-3 induced Bcl-x gene expression. Regulation of Bcl-x mRNA expression by IL-3 was reported to be inhibited in Ba/F3 cells by a dominant negative MAP kinase kinase mutant or by an inhibitor of the PI-3 kinase-AKT pathway (Leverrier *et al*., 1997, Leverrier *et al*., submitted). Induction of Bcl-x gene expression by transfection of a ras protein was observed in other cell types (Kinoshita *et al*., 1995a). These data suggest that coordinated activation of distinct signaling pathways are required for the regulation of Bcl-x gene expression in response to cytokines. Cross-talk between the JAK/STAT and the Ras/MAP kinase pathways has been described, and MAP kinase has been shown to regulate the transcriptional activity of STAT3 and STAT5A by serine phosphorylation (Wen *et al*., 1995; David *et al*., 1995; Pircher *et al*., 1997). The role of the MAP kinase on the serine phosphorylation of STAT5 in Ba/F3 cells after IL-3 stimulation remains to be elucidated however. Alternatively, induction of Bcl-x gene transcription might require the functional cooperation of STAT5 and other transcription factors that could be activated by the Ras/MAP kinase or the PI-3kinase-AKT pathways. Additional experiments are therefore needed to characterize in more details the Bcl-x gene promoter and to identify other potential transcription factors that could functionally interact with STAT5.

Finally, our data support the role of STAT5 in the protection from apoptosis and suggest that the transcriptional regulation of Bcl-x gene expression by STAT5 might explain its anti-apoptotic effect.

Materials and methods

Cell culture and plasmids

IL-3 dependent Ba/F3 cells were grown in RPMI 1640 medium (Life Technologies) containing 4% Wehi supernatant, 10% fetal calf serum (Biological Industries), L-glutamine 2 mM (Life Technologies) and penicillin-streptomycin (10 U/ml and 10 μg/ml respectively: Life Technologies) at 37°C with 5% CO_2.

Ba/F3 cells were starved at a density of 0.4×10^6 cells/ml for 16 h in the absence of Wehi supernatant. Stimulations were performed with 10 ng/ml recombinant murine IL-3 (Preprotech Inc).

Plasmids pXM-STAT5A, pXM-STAT5B, TK-luciferase and the (STAT5)x6TK luciferase were described elsewhere (Moriggl *et al*., 1996). The plasmid pRSV–STAT5AΔ749 was made by ligation of a *Kpn*I–*Not*I fragment containing the STAT5AΔ749 insert from pXM-STAT5AΔ749 into the pRSV-neo vector (*in vitro* gene). The promoter region of the human Bcl-x gene was obtained by PCR, using human genomic DNA as a template. The primers 5′-agacgagctccg-catttgttgggggtctccg-3′ and 5′-aatcgaaagcttcttcagtggactctgaat-ctcc-3′ were used for the amplification. The 1 Kb product was digested with *Sac*I and *Hind*III and cloned into the luciferase pGL2 vector to create the Bcl-x luciferase construct (Promega). Point mutation in the STAT5 binding site has been introduced by using a two steps PCR reaction.

Electroporation and cell cloning

The pRSV–STAT5AΔ749 (10 μg) or pRSV-neo (10 μg) plasmids were electroporated in Ba/F3 cells (250 V, 960 μF). Cells were expanded for 24 h before the G418 selection (1 mg/ml). Pools of G418 resistant cells were cloned by limited dilution.

Measurement of viability

Ba/F3neo and Ba/F3Δ749 cells were cultured in RPMI 10% fetal calf serum and 4% of WEHI-3B conditioned medium as a source of IL-3. To remove IL-3, cells were washed twice in RPMI 10% FCS. 0.1×10^6 cells were harvested at the time indicated and washed in cold PBS, fixed and permeabilized with ethanol 70% for 10 min at −20°C. Fixed cells were washed twice in cold PBS. The cells were resuspended in PBS with RNaseA (1.3 mg/ml, Sigma Chemical Co. St. Louis, MO, USA) and propidium iodide (33 μg/ml, Sigma) and incubated at room temperature for 15 min. The percentage of viability was analysed on an Epics Elite ESP cytometer (Coulter Corporation, Florida, USA).

DNA fragmentation assay

2×10^6 cells were lysed in Tris/HCl 10 mM pH 8, EDTA 100 mM, EGTA 10 mM, SDS 0.5%, RNaseA 4 mg/ml for 2 h at 37°C and then the extract was treated with proteinase K 100 μg/ml for 1 h at 56°C. Genomic DNA was recovered by phenol/chloroform extraction and ethanol precipitation. DNA resuspended in TE were analysed on agarose gel.

Antibodies and Western blotting

Whole cell extracts or NP40 lysates were separated by electrophoresis on SDS–PAGE and blotted onto cellulose membrane (Hybond-C super membrane, Amersham Life Science). Blots were incubated as indicated with antibodies raised against Bcl-2, Bax (Santa Cruz), Bcl-x or STAT5

(Transduction Laboratories). The anti STAT5A, anti STAT5B and anti STAT5 NH2-terminal antibodies used in supershift experiments were already described (Gouilleux *et al*., 1995; Moriggl *et al*., 1996).

Northern blotting

Total RNA were prepared from 8×10^6 Ba/F3neo or Ba/F3Δ749 cells using Trizol reagent method (Life Technologies). Twenty μg of total RNA were denatured, size-fractionated by phosphate-agarose gel electrophoresis and blotted onto a nylon filter (Hybond-N^+ membrane, Amersham Life Science).

The filter was hybridized with ^{32}P-labeled cDNA and detected by autoradiography. The probes used were a 900 bp human Bcl-x cDNA fragment, a fragment of the murine CIS cDNA and a 1.5 kb fragment from the human glyceraldehyde-3-phosphate dehydrogenase cDNA (GAPDH).

Reverse transcription (RT)–PCR assay

Total RNA (2 μg) was subjected to first-strand cDNA synthesis using Hexa random primer (Pharmacia Biotech) and Moloney murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies). PCR was performed using 0.5 μg of reverse transcript as a template. The 5′ primer and the 3′ primer used for Bcl-x amplification were 5′-AGGACTGAGGCCCCAGAA-3′ and 5′-CTACGGGAA-CAATGCAGCA-3′, respectively. PCR (1 min at 94°C, 1 min at 50°C, 1 min at 72°C) was performed during 35 cycles. PCR for actin was provided at 16 cycles (45 s at 94°C, 45 s at 50°C, 45 s at 72°C) using reverse transcript (0.1 μg) and the primers 5′-GAGACCTTCAACACCCC-3′ and 5′-GTGGTGGTGAAGCTGTAGCC-3′. The PCR products were separated by 1.5% agarose gel electrophoresis and blotted onto a nylon membrane (Hybond-N^+ membrane, Amersham Life Science). Membrane was probed with ^{32}P labeled Bcl-x oligonucelotide and actin cDNA fragment.

Whole cell extracts and electrophoretic mobility shift assays

Whole cell extracts were prepared by suspension of cell pellets in a buffer containing 400 mM NaCl, 50 mM KCl, 20 mM HEPES pH 7.9, 1 mM EDTA, 20% glycerol, 1 mM DTT, 0.2 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml apotinin and 100 μM of sodium orthovanadate. After three freezing–thawing cycles, the cells were centrifuged for 15 min at 4°C and 14 000 r.p.m. and the supernatants were recovered for bandshift or Western blotting experiments. The protocol for the bandshift assays has been previously described (Wakao *et al*., 1994). The MGF-STAT5 binding site from the bovine β-casein gene promoter (5′-AGATTTCTAGGAATTCAAATC-3′) or the STAT binding element (−336; −316) of the Bcl-x gene promoter (5′-GCATTTCGGAGAAGACGGGG-3′) were end-labeled with polynucleotide kinase to a specific activity of 8000 d.p.m./fmol and used as probes.

Plasmids, transient transfections and luciferase assays

Twenty μg of the TK promoter luciferase or of the (STAT5)x6TK luciferase constructs were electroporated in Ba/F3neo or STAT5AΔ749 cells. Twenty μg of the Bcl-x luciferase or the point mutated Bcl-x-luciferase constructs with 20 μg of PXM vector or 20 μg of PXM-STAT5 plasmids (10 μg of PXM-STAT5A and 10 μg PXM-STAT5B plasmids) were used in transient transfection of parental Ba/F3 cells. Cells were stimulated with 10 ng/ml of IL-3 for 16 h. Total cell extracts were prepared and used for luciferase activities according to the manufacturer's instructions (Promega kit).

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