Erythropoietin Can Induce the Expression of Bcl-xL through Stat5 in Erythropoietin-dependent Progenitor Cell Lines*

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Erythropoietin (Epo) initiates its cellular response by binding to the Epo receptor, which triggers the activation of signal transducer and activator of transcription (Stat) 5 protein. Cell culture studies of erythroid progenitors have suggested that Epo functions as a survival factor by repressing apoptosis at least in part through Bcl-xL, an anti-apoptotic protein of the Bcl-2 family. In this report, we examine whether Stat5 can induce transcription of the bcl-x gene in response to Epo. Two Epo-responsive progenitor cell lines, HCD-57 and Bel-2-transfected Ba/F3-Epo receptor (Ba/F3-EpoR-Bcl-2), were used in this study. After Epo stimulation, we observed a correlation between expression of bcl-xL and activation of Stat5 as assessed by the expression of oncostatin M, a direct target of Stat5, and the phosphorylation and nuclear translocation of Stat5. Moreover, a Stat binding element in the bcl-x promoter was found to be active in response to Epo, a finding that was further confirmed because mutagenesis of this sequence motif abrogated its promoter activity and overexpression of a dominant negative Stat5 protein blocked transactivation. When DNA-protein binding analyses were performed, we found that Stat5, not Stat1 or Stat3, was the protein bound to the bcl-x promoter in response to Epo. These data suggest that Epo-dependent activation of Stat5 is a transcriptional pathway that can be used by Epo-responsive progenitor cells to induce the expression of bcl-xL and consequently to inhibit apoptosis.

The intracellular molecular pathways that regulate the survival of primitive erythroid progenitors are still poorly understood. Erythropoietin (Epo)1 is necessary for erythroid progenitor proliferation and to prevent the apoptotic cell death of immature erythroblasts (1, 2). Epo binds to a specific cell surface receptor that is expressed on erythroid progenitors (3, 4). The Epo receptor associates with Jak2, a member of a subfamily of protein tyrosine kinases, which plays an important role in cytokine-dependent gene regulation. Activated Jak2, in turn, converts a latent cytoplasmic transcription factor, Stat5, into its active form by tyrosine phosphorylation. The activated Stat5 translocates into the nucleus, where it binds to specific DNA response elements in the promoter region of target genes and activates transcription (5–7).

It has been shown that the ability of interleukin-2 to signal activation of Stat5 in 32D lymphocytes is directly related to its ability to promote protection from apoptosis and long-term cell growth (8). Furthermore, growth factor-independent activation of the Jak2-Stat5 pathway in Nb2 lymphocytes protects these cells against apoptosis induced in the absence of growth factor (9).

We have recently shown that a member of the Bcl-2 family of apoptosis-regulatory proteins, Bcl-xL (10), but not Bcl-2, is highly expressed in the erythroleukemic cell lines HEL and K562 (11) and that the constitutive expression of Bcl-xL protects cells from apoptosis induced by differentiation inducers. Furthermore, Epo maintains survival and represses apoptosis of HCD-57 erythroid progenitor cells through the expression of Bcl-xL (12). This anti-apoptotic protein is down-regulated after Epo withdrawal in HCD-57 cells, and the cells undergo apoptotic cell death. Interestingly, when HCD-57 cells transduced with a retroviral vector encoding Bcl-xL are cultured in the absence of Epo, the endogenous level of Bcl-xL is down-regulated, but the cells remain viable, further indicating that the expression of Bcl-xL is mediated by Epo and that this is an important mechanism to repress apoptosis in erythroid progenitors. The relevance of this association has been suggested in patients with polycythemia vera, in whom autonomous Epo-independent erythroid cells arise from monoclonal progenitors. These abnormal progenitor cells exhibit a deregulated expression of Bcl-xL that may contribute to bypass the need for the growth factor and may consequently explain their Epo-independent survival (13).

The genomic organization and promoter region of the bcl-x gene have recently been determined (14), which facilitates the analysis of the transcriptional factors involved in the expression of bcl-x. In the present study, we have examined whether Epo induces the expression of bcl-xL through activation of Stat5 to better understand the molecular basis for the survival of erythroid progenitor cells. We have identified an Epo-responsive motif for the binding of a Stat protein (Stat binding element) in the untranslated 5’ region of the mouse bcl-x gene. Furthermore, we have shown that Stat5 binds to the Epo-responsive motif and that this motif is active in response to Epo because transient transfection experiments showed activity of a reporter gene in the presence of Epo that can be abrogated by mutagenesis of the Stat binding element or overexpression of a
dominant negative Stat5 protein. These findings suggest that at least one of the molecular pathways that maintains the survival of erythroid progenitors may be triggered by the interaction of Epo with its specific receptor, which induces the expression of Bcl-xL through the binding of Stat5 to the bcl-x promoter.

EXPERIMENTAL PROCEDURES

Cell Culture—The murine Epo-dependent HCD-57 cell line was maintained in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc.) as described previously (12). Ba/F3 cells stably expressing the Epo receptor (15) were transfected by electroporation (960 microfarads; 250 V) with 20 μg of the pSFFV-Neo plasmid containing bcl-2. Individual cell clones were selected for growth in the presence of G418 (1 mg/ml) and hygromycin B (1 μg/ml) by limiting dilution. Three individual clones expressing high levels of Bcl-2 were randomly selected and used by virtue of resistance to apoptosis. Ba/F3-EpoR-Bcl-2 clones were grown in RPMI 1640 medium (Seromed Biotech KG, Berlin, Germany) supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, CA), 2 × 10⁻³ M 2-mercaptoethanol, 2 mM glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1 unit/ml recombinant murine Epo (Roche Molecular Biochemicals). In some experiments, Ba/F3-EpoR-Bcl-2 cells were starved of Epo for 24 h and then treated with 100 μM aurintricarboxylic acid (ATA) (Sigma) for 2 h.

mRNA Expression Analysis—Total RNA was prepared using TRIzol reagent (Life Technologies, Inc.). To assess mRNA expression, a semiquantitative reverse transcription-PCR method was used as described previously (12). The generated cDNA was amplified by using primers for murine bcl-x, bax (12), and oncostatin M (5′-TTGATTCAGGGGTCT-GATGAC-3′ and 5′-AAAATGTCATGTCCCTCCAAG-3′). The amplification profile was as follows: 94 °C for 30 s, 55 °C for 20 s, and 72 °C for 40 s. After 25 amplification cycles, the expected PCR products (344 base pairs for bcl-xL, 194 base pairs for bax, and 658 base pairs for oncostatin M) were size fractionated onto a 2% agarose gel and stained with ethidium bromide.

Western Blotting of Nuclear Lysates—Cells were cultured in the absence of Epo for 24 h and then stimulated for 30 min with Epo. For preparation of nuclear lysates, cells were lysed for 15 min on ice in lysis buffer (0.05% Nonidet P-40, 20 mM Tris, pH 8.0, 137 mM NaCl, 5 mM MgCl₂, 10 mM sodium molybdate, 10 mM 2-mercaptoethanol, 5 mM dithiothreitol, and 15 mM p-nitrophenylphosphosphate and protease inhibitors). After the samples were spun, the supernatants were used as nuclear extracts. The expression of Stat5 and GATA-1 proteins in the nuclear fraction was determined by Western blotting as described previously (11). Blots were incubated with mouse anti-Stat5 (Transduction Laboratories, Lexington, KY) or rat anti-GATA-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated with goat anti-mouse or goat anti-rabbit antibodies conjugated to alkaline phosphatase (Tropix, Bedford, MA). Bound antibody was detected by a chemiluminescence system (Tropix).

Immunoprecipitation—Cells were cultured in the absence of Epo for 24 h and then stimulated for different time intervals with Epo and lysed in 0.5% Nonidet P-40, 50 mM Tris, pH 8.0, 0.1 mM EDTA, 150 mM NaCl, and 1 mM diethiothreitol and protease inhibitors. Lysates were cleared of debris, and the supernatants were incubated with rabbit anti-Stat5b conjugated to agarose beads (Santa Cruz Biotechnology), which recognizes both Stat5a and Stat5b. Proteins eluted from the agarose beads were electrophoresed and transferred to nitrocellulose as described previously (11). Membranes were incubated with mouse anti-Stat5 or mouse anti-phosphotyrosine (Transduction Laboratories), and antibodies were detected by chemiluminescence (Tropix).

Electrophoretic Mobility Shift Assays—Ba/F3-EpoR-Bcl-2 cells were cultured in the absence of serum and Epo for 24 h and then stimulated with 100 units/ml Epo for 30 min. Cells were lysed in 0.6% Nonidet P-40, 10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 1 mM diethiothreitol, and 10 mM sodium molybdate and protease inhibitors. Whole cell lysates were spun, and the nuclear fractions were resuspended in 20 mM HEPES, pH 7.6, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM diethiothreitol, and 10 mM sodium molybdate and protease inhibitors. As described elsewhere (16), nuclear extracts (5 μg of total protein) were incubated with a 32P-labeled double-stranded DNA probe (5′-TTGATTCAGGGGTCTTGATGAC-3′) from the promoter region of the mouse bcl-x gene (14), the bcl-x probe carrying a mutated Stat binding site, or a β-casein probe, which contains a consensus binding site for Stat5 (5′-AGATTCTAGGATATCATTCC-3′). Samples were run on a 5% nondenaturing polyacrylamide gel in 20 mM Tris base, 2 mM EGTA, 5 mM MgCl₂, 10 mM sodium fluoride, 0.1 mM M 2-mercaptoethanol, 2 mM glutamine, 100 units/ml aprotinin, and 5 μM leupeptin (Promega). Gels were dried and visualized by autoradiography. Supershifts were performed using rabbit polyclonal antibodies specific for Stat5, Stat1, or Stat3 proteins (Santa Cruz Biotechnology). For competition assays, nuclear extracts containing equal amounts of total protein were pre-incubated with a 100-fold molar excess of either unlabeled bcl-x probe or unlabeled irrelevant DNA fragment.

Gene Reporter Assays—Ba/F3-EpoR-Bcl-2 cells were cultured in the absence of Epo for 24 h and then transfected by electroporation (270 V; 960 microfarads) with 50 μg of a luciferase reporter vector containing a 0.6-kb fragment of the bcl-x promoter (pGL2-0.6R) before adding Epo. Total RNA was obtained at the indicated intervals and analyzed for oncostatin M, bcl-xL, and bax mRNA levels by semiquantitative reverse transcription-PCR. After 25 amplification cycles, PCR products were electrophoresed onto a 2% agarose gel and stained with ethidium bromide.

RESULTS

Expression of bcl-xL Correlates with Activation of the Jak2-Stat5 Pathway—We have previously shown that Epo can function as a survival factor by repressing apoptosis through the expression of Bcl-xL (12). Because Epo induces the expression of a number of genes through activation of the Jak2-Stat5 pathway (18), we first studied whether the expression of bcl-xL correlated with the expression of oncostatin M, a member of the interleukin-6-related cytokine subfamily induced by Epo and a direct target of Stat5 (19). When two Epo-responsive cell lines, HCD-57 and Ba/F3-EpoR-Bcl-2, were cultured in the absence of Epo, the levels of oncostatin M and bcl-xL mRNA were downregulated by 24 h after Epo withdrawal as assessed by semiquantitative reverse transcription-PCR analysis (Fig. 1). Re-
addition of Epo to growth factor-starved cells up-regulated the expression of both bcl-xL and oncostatin M. As shown in Fig. 1, the mRNA levels of bcl-xL increased 1 h after the addition of Epo and continued to accumulate. At 16 h, the expression of bcl-xL was similar to that of control cells. The levels of oncostatin M mRNA started to increase within 15 min and reached a maximal level of expression at 30 min to 1 h, decreasing thereafter to baseline levels. To study whether the induction of bcl-xL requires de novo protein synthesis, Epo-starved cells were pretreated with 20 μg/ml cycloheximide for 15 min, a dose that blocked more than 90% of protein synthesis within 15 min as determined by [35S]methionine incorporation analysis (data not shown). As shown in Fig. 1, oncostatin M mRNA levels were further elevated in cells treated with cycloheximide plus Epo relative to cells treated with Epo alone. In addition, a modest hyperinduction of bcl-xL mRNA was also observed in cells treated with cycloheximide plus Epo. Therefore, Epo induces the early expression of bcl-xL and oncostatin M in HCD-57 and Ba/F3-EpoR-Bcl-2 cells, and although bcl-xL followed a kinetics delayed relative to the rapid induction of oncostatin M, both genes are induced in a protein synthesis-independent manner. In contrast, the steady-state mRNA levels of bax, another bcl-2-family member that promotes apoptosis (20), were not regulated by Epo stimulation or by treatment with cycloheximide plus Epo. The activation of a Jak2-Stat5 signaling pathway by ATa, a triphenylmethane derivative with anti-apoptotic properties, has been recently described in Nb2 lymphocytes (9). ATa, a triphenylmethane derivative with anti-apoptotic properties, requires protein synthesis for its action. To study whether the induction of bcl-xL was mediated by Stat5, we treated Ba/F3-EpoR-Bcl-2 cells with oncostatin M, and although oncostatin M increased 1 h after the addition of oncostatin M, the expression of both bcl-xL and oncostatin M was similar at all time points, indicating that the detection of phosphorylated Stat5 correlated with Epo stimulation (Fig. 3A). Because tyrosine phosphorylation of Stat5 is normally followed by dimerization and nuclear translocation, we also examined the presence of Stat5 in the nucleus of Ba/F3-EpoR-Bcl-2 cells. As shown in Fig. 3B, Stat5 was translocated to the nucleus only in response to Epo, as assessed by Western blotting with an anti-Stat5 antibody. Stripping the membrane and reprobing with antibody against the nuclear factor GATA-1 showed that protein loading was similar in the samples (Fig. 3B); consequently, the differences in nuclear expression of Stat5 were due to the stimulation with Epo. Although the results shown in Figs. 1–3 were obtained with a single Ba/F3-EpoR-Bcl-2 clone, similar conclusions were reached with two other clones (data not shown).

**Promoter Region of the bcl-x Gene Contains an Epo-responsive Motif**—The promoter region of the mouse bcl-x gene has recently been analyzed (14). This region contains a sequence upstream of the translation initiation codon in exon 2 consistent with a Stat binding element known as the interferon-γ activation site (GAS) (7). As shown by transfection with reporter constructs, the constitutive activity induced by a 3.2-kb genomic fragment upstream of exon 2 was mainly contained within a 0.6-kb 3′ sequence (0.6R) (Ref. 10; Fig. 4A). To study the promoter activity of this fragment, which contained the Stat binding element, a 0.6R luciferase vector was transiently transfected into Ba/F3-EpoR-Bcl-2 cells. The constitutive expression of Bcl-2 allows these cells to survive in the absence of Epo during a 20–24-h period, which was a critical step before the stimulation with Epo that minimized the activation of factors involved in Epo-mediated signal transduction. The same analysis was performed in three independent Ba/F3-EpoR-Bcl-2 clones, which yielded similar results (data not shown). Fig. 4 shows a representative experiment with one of these clones. The levels of luciferase activity detected in the Epo-stimulated cells were significantly higher than those observed in unstimulated cells (>2.5-fold) (Fig. 4B). As a control, another construct containing a 0.6-kb 5′ fragment (0.6L) upstream of the 0.6R fragment was analyzed for luciferase activity in response to Epo. As shown in Fig. 4B, the luciferase activity detected in Ba/F3-EpoR-Bcl-2 cells transfected with a 0.6L luciferase vector was low in both the Epo-stimulated and unstimulated cell populations. When we examined the entire 1.2-kb fragment (0.6L and 0.6R), we found that the luciferase activity was similar to that obtained with the 0.6R fragment (data not shown). To further clarify the functional specificity of the Stat binding element contained in the 0.6R fragment, a mutagenesis analysis was performed. We mutated three bases within this sequence motif (normal, TCTGGAGA/A mutant, TGAGGATAA) and transfected Ba/F3-EpoR-Bcl-2 cells with a 0.6R mutant luciferase vector. As shown in Fig. 4B, the levels of luciferase activity were significantly lower than those ob-
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**Fig. 4.** Transcriptional activation of a luciferase reporter construct driven by the bcl-x promoter in response to Epo. A, schematic representation of the bcl-x promoter region that contains the fragments 0.6L and 0.6R used in this study. B, Ba/F3-EpoR-Bcl-2 cells were co-transfected with a constitutive luciferase reporter vector (pRL-TK) and the following constructs: 0.6L luciferase, 0.6R luciferase alone or with pSFFV-Oct-1750, and 0.6R mutant luciferase in which the Stat binding element has been mutated. Cells were induced with Epo for 16 h or left untreated. Units of luciferase activity were normalized for Stat binding element has been mutated. Cells were induced with Epo (18). To study whether Stat5 mediates the effect of Epo on the transcription of genes in erythroid cells, we analyzed two Epo-responsive motifs of the bcl-x promoter in erythroid cells, we analyzed two Epo-responsive motifs of the bcl-x promoter (19, 30). 

**Fig. 5.** Binding of Stat5 to the bcl-x promoter in response to Epo. Bcl-xL receptor expressed on the surface of immature erythroblasts (3, 4). After ligand binding, EpoR is known to activate a cytoplasmic protein tyrosine kinase, Jak2, which in turn activates a transcription factor, Stat5, triggering a signal transduction cascade that leads to the development of early erythroid progenitors into mature erythroblast cells (22). In the absence of Epo, erythroid progenitors die, and their genomic DNA is degraded into oligonucleosomal fragments, a feature of apoptotic cell death (1, 2). Several members of the bcl-2 family of apoptosis-regulatory genes that function as inhibitors of apoptosis in hematopoietic cells have been identified (10, 23). We have recently demonstrated that when the Epo-dependent erythroid progenitor cell line HCD-57 is cultured in the absence of Epo, the expression of Bcl-x<sub>L</sub> is rapidly down-regulated, and this is accompanied by the activation of an apoptotic process (12). The constitutive expression of Bcl-x<sub>L</sub> rescues erythroid progenitors from apoptosis induced by Epo deprivation, suggesting that Epo can function as a survival factor by repressing apoptosis through Bcl-x<sub>L</sub> in erythroid progenitor cells. In an attempt to identify the signaling pathway involved in the transactivation of the bcl-x promoter in erythroid cells, we analyzed two Epo-dependent cell lines, HCD-57 and Ba/F3-EpoR-Bcl-2. HCD-57 is a murine erythroid progenitor cell line that undergoes erythroid differentiation in the presence of hemin (12). Ba/F3-EpoR is a murine progenitor cell line stably transfected with the Epo receptor that proliferates and undergoes limited erythroid differentiation in Epo (15, 24). We have transfected this cell line with bcl-2 (Ba/F3-EpoR-Bcl-2) to avoid apoptosis when cultured in the absence of Epo. The early induction of bcl-x mRNA after Epo stimulation followed an accumulation pattern that was maintained or even enhanced in the presence of cycloheximide.
A protein synthesis-independent induction was also shown for oncostatin M, an early response gene induced by Epo through the Jak-2-Stat5 pathway. It has been noted previously (25) that bcl-xL behaves as a delayed early response gene in that it requires de novo protein synthesis during liver regeneration. However, it is important to consider that the bcl-x promoter contains characteristic motifs for the binding of several transcription factors, including Ets-1, AP4, NF-E2, Evi-1, GATA-1, and AP-1 (14), which suggests that bcl-xL may be induced by different transcriptional pathways distinguished by their requirement for de novo protein synthesis, among other features. Because a Stat binding element has been found in the promoter region of the bcl-x gene (26), we studied the promoter activity of this sequence motif in response to Epo in Ba/F3-EpoR-Bcl-2 cells. We found that the Stat binding element responded to Epo by promoting the expression of a reporter gene and that this sequence specifically bound Stat5 as assessed by DNA-protein binding analysis. The observation that the Jak-Stat signaling pathway seems to be necessary to prevent apoptosis in erythroid progenitors in response to Epo (27) is consistent with this finding. It has been shown that the Epo-dependent inhibition of apoptosis is blocked by the ectopic expression of kinase-deficient dominant negative forms of Jak2, suggesting an essential role for this tyrosine kinase in the apoptotic pathway (28). We have activated the Jak-Stat pathway by treating Ba/F3-EpoR-Bcl-2 cells with ATA. This compound mimics growth factor-induced tyrosine phosphorylation of Jak2 and activation of Stat5, resulting in the induction of Stat5-regulated genes (9). Interestingly, ATA does not seem to regulate other members of the Stat family, nor does it affect Jak3. Consistent with this specificity, we found that ATA mimicked Epo-induced expression of oncostatin M, a direct target of Stat5 (19), and bcl-xL. These data suggest a model in which Epo triggers the activation of the Jak2-Stat5 transduction pathway, and Stat5 induces the expression of bcl-xL that blocks the apoptotic machinery of Epo-responsive progenitor cell lines. Very recently, it has been shown that the phenotypes of mutant mice that have the Stat5 genes deleted demonstrate an essential role for these proteins in physiological responses associated with growth hormone and prolactin, whereas the responses to a variety of cytokines, including Epo, are largely unaffected (29). However, the expression of bcl-xL was not analyzed in any cellular compartment. It is likely that in these deficient mice, the expression of bcl-xL is diminished or abrogated, and other members of the Bcl-2-family promote erythroid progenitor survival. Consistent with this finding, it has been shown that HCD-57 erythroid cells express both Bcl-xL and Bcl-2, which are down-regulated after Epo withdrawal (12), and that although Bcl-xL seems to be the predominant anti-apoptotic protein in this cell line, Bcl-2 may contribute to cell survival. Consequently, transcription factors other than Stat5 may induce the expression of survival genes (i.e. bcl-2) in response to Epo. Alternatively, it is possible that other transcription factors may induce the expression of bcl-x. In fact, it has been shown that Stat1 mediates the expression of bcl-x in cardiac myocytes after induction with leukemia inhibitory factor (26); however, we have shown that neither Stat1 nor Stat3 was able to bind the Stat-binding element from the bcl-x promoter. Consistent with these data, it has been described that in human primary erythroid precursors, only Stat5, not Stat1 or Stat3, is activated after stimulation with Epo (30), which makes it unlikely that other Stat proteins might transactivate bcl-x in erythroid cells. A more likely candidate might be GATA-1 because a consensus motif for transcription factors of the GATA family has been identified in the promoter region of bcl-x (14). GATA-1 has been shown to be involved in the regulation of erythroid progenitor survival and differentiation by preventing apoptosis (31). In conclusion, our data indicate that Epo-mediated activation of Stat5 can induce the expression of bcl-x in Epo-responsive progenitor cell lines; although there may be other transcriptional pathways involved in this process, we show for the first time a transactivation mechanism of the bcl-x promoter induced by Epo, which may account at least in part for the anti-apoptotic activity of Epo in erythroid cells.

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