Thrombopoietin (TPO), an essential factor for megakaryopoiesis and thrombopoiesis, works as a survival factor for megakaryocytic lineage cells. However, little is known about the molecular mechanism in detail. We show here that TPO supports the survival of TPO-dependent leukemia cell line UT-7/TPO and normal megakaryocytic progenitors via the induction of Bcl-xL, an anti-apoptotic member of the Bcl-2 family. We further analyzed the signal transduction pathways required for TPO-induced Bcl-xL gene expression. A reporter assay with various lengths of Bcl-x gene promoter revealed that both Stat- and nuclear factor κB-binding sites are prerequisites for TPO-induced promoter activity. Consistent with these results, TPO induced the binding of Stat5 and subunits of nuclear factor κB, p50, and c-Rel to the Bcl-x gene promoter. AG490, a specific inhibitor for Jak2, and LY294002, a specific inhibitor for phosphatidylinositol (PI) 3-kinase, reduced the protein level of Bcl-xL in UT-7/TPO cells, accompanied by an increase in the ratio of apoptotic cells. Interestingly, LY294002 enhanced the TPO-induced DNA binding activity of Stat5 without affecting the Jak2 activation and tyrosine phosphorylation of Stat5. Concomitantly, confocal microscopy revealed that LY294002 clearly inhibited the nuclear export of Stat5, suggesting that PI 3-kinase regulates the subcellular localization of Stat5. Taken together, our results suggest that both Jak-Stat and PI 3-kinase activation pathways regulate the TPO-induced survival of megakaryocytic cells via Bcl-xL gene expression. In addition, our data suggest possible cross-talk between these two signaling pathways.

Thrombopoietin (TPO) is the major regulator of megakaryopoiesis and thrombopoiesis, leading to platelet production (1, 2). One of the most important functions of TPO is to support the survival of megakaryocytic lineage cells. Withdrawal of TPO causes the apoptosis of both TPO-responsive cell lines (3) and in immature megakaryocytic progenitor cells (4). An animal model study demonstrated that disruption of the TPO gene or its receptor c-mpl gene causes severe thrombocytopenia (5, 6). Furthermore, point mutations of c-mpl gene were found in some patients with congenital amegakaryocytic progenitor cells (5, 6). Although it is well recognized that TPO is essential for the survival of megakaryocytic lineage cells, the precise molecular mechanism of how TPO prevents megakaryocytic progenitor cells from apoptosis is still open to question.

Bcl-2 family proteins play key roles in the control of programmed cell death by hematopoietic cytokines (7). First, hematopoietic cytokines induce the expression of anti-apoptotic members of these proteins, such as Bcl-2 and Bcl-xL. These anti-apoptotic Bcl-2 proteins protect apoptosis through the inhibition of cytochrome c release from the mitochondria (7). Second, hematopoietic cytokines inhibit apoptosis through phosphorylation of BAD, a pro-apoptotic member of the Bcl-2 family. Phosphorylation of BAD causes altered intracellular distribution and leads to loss of pro-apoptotic function of this protein (8). Suppression of pro-apoptotic members of this family, known as BH3-only proteins, is another important function of cytokines to protect hematopoietic cells from apoptosis (9, 10). Thus, although all of these mechanisms are important for cytokine-mediated anti-apoptotic effects, it appears that Bcl-xL plays central roles as a downstream molecule of hematopoietic cytokine signaling. Several lines of evidence support this notion. Bcl-xL is expressed in both CD34+/CD38− multipotent hematopoietic stem cells (11, 12) and committed progenitors having erythroid (13, 14) or monocytic lineage properties (15). Genetic elimination of Bcl-xL genes causes severe deficiency of hematopoietic cells resulting from the increased ratio of apoptotic cells (16).

Bcl-xL expression is mainly regulated by hematopoietic cytokines through the activation of intracellular signal transduction pathways (17). Although a variety of signal transduction molecules are activated by hematopoietic cytokines, recent studies have revealed that Jak-Stat and PI 3-kinase activation pathways mainly regulate Bcl-xL gene expression. In the erythropoietin (EPO)-dependent cell line HCD-57, EPO induced the binding of Stat5 to the consensus Stat-binding sites on the Bcl-x gene promoter (18). Stat5 is also required for IL-3-dependent survival of Baf-3 cells (19). In contrast, IL-15 induced the binding of Stat6 to the Bcl-x gene promoter in mast cells (20). In addition to these in vitro studies, Stat5a and Stat5b knockout mice also demonstrated that Stat5 is required for Bcl-xL expression in erythroid lineage cells (21). Another important molecule for Bcl-xL regulation is PI 3-kinase. PI 3-kinase is a key molecule for controlling apoptosis in a variety of cell type. PI 3-kinase prevents cells from apoptosis through a...
varied with apoptosis. Recent reports have shown that PI 3-kinase is involved in the regulation of Bcl-xL expression. Leverrier et al. (22) reported that PI 3-kinase is required for IL-3- and insulin-like growth factor-1-induced Bcl-xL expression in BaF3 cells. Like these cytokines, TPO activates both Jak-Stat and PI 3-kinase pathways in TPO-dependent cell lines and primary megakaryocytic cells (23, 24). Furthermore, a recent study has indicated that megakaryocytic progenitor cells express Bcl-xL (25). Taken together, we hypothesized that TPO supports the survival of megakaryocytic progenitor cells through Jak-Stat and/or PI 3-kinase-dependent Bcl-xL expression.

In this study, we examined the role of Bcl-xL in TPO-dependent cell survival. Furthermore, we tried to clarify the role of both Jak-Stat and PI 3-kinase activation pathways in the regulation of Bcl-xL expression and subsequent apoptosis. We show here that Bcl-xL expression is dependent on TPO in TPO-dependent human leukemic cell line UT-7/TPO and megakaryocytic cells. We also show that TPO regulates Bcl-xL gene expression through Jak2-dependent activation of Stat5 and PI 3-kinase-dependent activation of NF-κB. Finally, we show that subcellular localization of Stat5 is in part regulated by PI 3-kinase activity.

**EXPERIMENTAL PROCEDURES**

**Cells**—UT-7/TPO cells were maintained in liquid culture with Iscove’s modified Dulbecco’s medium (Invitrogen) containing 10% fetal calf serum (FCS; HyClone Laboratories, Logan, UT) and 10 ng/ml TPO (26).

**Hematopoietic Growth Factors and Reagents**—Recombinant human TPO was provided by the Kirin Brewery Co. (Gumma, Japan). Neomyacin (G418) was purchased from Invitrogen. AG490 and LY294002 were purchased from Calbiochem (La Jolla, CA). Antiserum for p50, p65, and c-Rel of NF-κB subunits were purchased from Rockland (Gilbertsville, PA). Antiserum against Stat1α for the supershift analysis was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Antiserum against Stat3 and Stat5 for the supershift assay were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-phospho-Stat5 Jak2 and anti-phospho-Stat5 antibodies were purchased from BIOSOURCE (Camarillo, CA) and Cell Signaling (Beverly, MA), respectively. 

**Preparation of Cell Lysates, Immunoprecipitation, and Western Blotting**—Cell lysates were prepared from UT-7/TPO or normal megakaryocytic cells according to the methods previously described (26). The cell lysates were resolved by SDS-PAGE and then electroblotted onto a polyvinylidene difluoride membrane. The blots were incubated with an appropriate concentration of primary antibodies including anti-β-actin antibody (1:1,000; Sigma), anti-parafomaldehyde, and 0.4% Triton X-100 for 20 min at room temperature. After a wash with PBS, the cells were incubated with anti-Stat5 antibody diluted 1:200 in PBS containing 2% FCS and 0.05% Tween 20 at 4°C for 1 h and then transferred with liposome control plasmid pRL-TK-LUC (Promega, Madison, WI). The UT-7/TPO cells were cytokerine-starved for 12 h and then transfected with reporter DNAs. After transfection, the cells were cultured for 12 h without cytokines and stimulated with TPO (100 ng/ml) for 24 h, and the cells were harvested for dual luciferase assay according to the manual (Promega). In some experiments, the expression vectors, pcDNA3.1, pcDNA3.1-Stat3F, or pcDNA3.1-Stat5F were cotransfected.

**Detection of Apoptotic Cells**—Apoptotic cells were detected according to the manufacturer’s instructions. In brief, the cells were washed with phosphate-buffered saline (PBS) and resuspended in binding buffer. After 15 min of incubation with annexin V-fluorescein isothiocyanate and propidium iodide, the cell samples were measured by flow cytometry (FACSscan, Becton Dickinson, Franklin Lakes, NJ) using a single laser emitting excitation light at 488 nm.

**Ex Vivo Generation ofMegakaryocytic Cells**—Peripheral blood-derived CD34 cells were collected and expanded according to the methods previously described (29). The purified CD34+ cells were cultured with TPO for 10 days. Then, the cells were cultured with or without TPO for 24 h and harvested for annexin V expression analysis and Western blotting.

**Confocal Microscopy**—Cytospin preparations were fixed and permeabilized with 0.1% PBS containing 1% paraformaldehyde and 0.4% Triton X-100 for 20 min at 4°C. The double stained PI/Annexin V samples were analyzed using a TCS 4D confocal system (Leica Instruments, Wetzlar, Germany).

**RESULTS**

**Expression of Bcl-xL is Dependent on TPO in UT-7/TPO Cells**—To clarify the functional role of Bcl-xL and Bcl-2, belonging to the Bcl-2 family, in TPO-dependent cellular survival, we first analyzed the changes in expression level of these proteins after TPO deprivation in UT-7/TPO, which is dependent on TPO for growth and survival. As shown in Fig. 1, the UT-7/TPO cells underwent apoptosis after 24-h deprivation of TPO. The amount of Bcl-xL proteins was reduced after 24-h starvation and completely disappeared after 48 h (Fig. 1B). In contrast, the Bcl-2 protein levels were unchanged even after 48-h starvation. Next, we analyzed whether TPO treatment actually induces the Bcl-xL expression in UT-7/TPO cells. After 24-h starvation, UT-7/TPO cells were cultured with TPO for the indicated periods and then the Bcl-xL expression was analyzed. The amount of Bcl-xL proteins was increased after 12-h TPO stimulation (Fig. 1C). In contrast, TPO did not affect the expression of Bcl-2. To confirm the Bcl-xL expression level of BC-7-27Bm, these results indicated that TPO-induced cell survival is correlated with Bcl-xL but not Bcl-2 expression in UT-7/TPO cells.

**TPO Supports Bcl-xL Expression in Normal Megakaryocytic Cells**—To extend the above notion to normal megakaryocytes, we examined the effect of TPO deprivation on the expression of Bcl-xL and Bcl-2 using normal human megakaryocytic cells. To
obtain a large number of megakaryocytes for Western blotting analysis, we cultured human CD34-positive cells in the presence of TPO for 10 days. More than 90% of the isolated human megakaryocytic cells expressed the specific megakaryocytic markers CD41 and CD61, and 45% of the cells were CD41/CD42b double-positive. Using these highly purified megakaryocytes, we performed Western blotting analysis with anti-Bcl-2 or anti-Bcl-xL antibody. As shown in Fig. 2A, the megakaryocytic cells expressed both Bcl-xL and Bcl-2 proteins. TPO deprivation reduced the Bcl-xL expression level, accompanied by an increased ratio of annexin V-positive cells (Fig. 2, A and B). In contrast, the Bcl-2 protein level was not affected for 24 h after TPO starvation (Fig. 2A). These results indicated that Bcl-xL but not Bcl-2 is involved in TPO-dependent survival of normal megakaryocytic cells.

Consensus NF-κB- and Stat-binding Sites Are Important for TPO-induced Bcl-x Gene Promoter Activity—Next, we examined whether or not TPO regulates the Bcl-xL expression at the transcriptional level. For this purpose, we used mouse Bcl-x gene promoter constructs fused to a luciferase reporter gene. This construct contains a 3.2-kb genomic fragment upstream of the translation initiation codon of the mouse Bcl-x gene. We introduced this construct into UT-7/TPO cells and assayed the luciferase activity with or without TPO stimulation. As illustrated in Fig. 3, treatment with TPO enhanced the activity of the Bcl-x gene promoter 2.7-fold compared with the FCS treatment. The magnitude was almost the same as other cytokines (18–21). Our results indicated that TPO regulates the Bcl-x gene expression through transcriptional control of the Bcl-x gene promoter. To investigate the precise regulatory region required for the TPO-induced Bcl-x gene promoter activity, we prepared a series of mutant reporter plasmids containing a variety of lengths of the Bcl-x gene promoter as illustrated in Fig. 3. We also introduced these mutant reporter plasmids into UT-7/TPO cells and performed a luciferase assay. A proximal 0.6-kb part of the Bcl-x gene promoter region was critical for TPO-induced reporter activation. Because this region contains consensus NF-κB- and Stat-binding sites, we hypothesized that NF-κB and/or Stat proteins may work as critical regulators for

**Fig. 1.** Effect of TPO deprivation on survival of UT-7/TPO cells and expression of Bcl-2 family proteins. A, effect of TPO deprivation on survival of UT-7/TPO cells. UT-7/TPO cells were washed and then cultured without TPO for the indicated periods. The ratio of apoptotic cells was analyzed by annexin V-fluorescein isothiocyanate staining and flow cytometer. The percentage of apoptotic cells is indicated. B, expression of Bcl-xL and Bcl-2 after TPO deprivation. After deprivation of TPO for the indicated periods, the cells were harvested for the preparation of total cell lysates. The expression level of Bcl-xL and Bcl-2 was analyzed by Western blotting analysis. C, expression of Bcl-xL and Bcl-2 after TPO stimulation. Starved UT-7/TPO cells were stimulated with TPO (10 ng/ml) for the indicated periods. The cells were then harvested for Western blotting analysis with anti-Bcl-xL or anti-Bcl-2 antibody.

**Fig. 2.** Bcl-xL expression in normal megakaryocytic cells. Expanded normal megakaryocytic lineage cells were cultured with or without TPO for 24 h and then harvested for Western blotting analysis with anti-Bcl-xL or anti-Bcl-2 antibody (A) or for analysis by annexin V staining (B).
the Bcl-x gene promoter activity in response to TPO.

**Stat5 Binds the Consensus Stat-binding Sequence on Bcl-x Gene Promoter**—Based on these observations, we analyzed the role of the Jak-Stat pathway in the regulation of Bcl-xL gene expression. We performed EMSA to investigate whether Stat proteins can bind to the Bcl-xL gene promoter after TPO stimulation, using oligonucleotides corresponding to the Stat-binding motif on the human Bcl-xL gene promoter as probes (21). As shown in Fig. 4A, TPO clearly induced the formation of DNA-binding complexes in UT-7/TPO cells. Preincubation of the nuclear extracts with a 150-fold molar excess of unlabeled probe inhibited the formation of these DNA binding complexes. To confirm that the DNA binding complex contains Stat proteins, we performed a supershift study with antibodies against Stat1, Stat3, or Stat5. After addition of anti-Stat5 antibody to the nuclear extracts, the DNA binding complex was supershifted (Fig. 4B). In contrast, neither anti-Stat1 nor anti-Stat3 antibodies had any effects on gel shift retardation. These results indicated that Stat5 specifically binds to the Bcl-xL gene promoter after TPO stimulation.

**Jak-Stat Pathway Is Required for Bcl-xL Gene Expression**—For further investigation, we used AG490, known as a Jak2 inhibitor (30). Treatment with 50 μM AG490 reduced both the Stat5 activation and Bcl-xL expression (Fig. 5, A and B). In addition, AG490 clearly caused apoptosis in approximately half of the AG490-treated UT-7/TPO cells at 50 μM (Fig. 5C). To confirm that Stat5 is actually involved in the activation of the Bcl-xL gene promoter, we used a dominant negative form of Stat5 in which the tyrosine residue required for dimer formation was mutated to phenylalanine. As shown in Fig. 6, a dominant negative form of Stat5 clearly inhibited the TPO-induced Bcl-xL gene promoter activity. On the other hand, a dominant negative form of Stat3 or vector alone did not affect the promoter activity. These results indicated that Stat5 is required for the TPO-induced Bcl-xL gene promoter activation.

**TPO Induced NF-κB Binding on Bcl-x Gene Promoter**—In addition to the Stat-binding motif, the TPO-responsive region on the Bcl-x gene promoter also contains the putative NF-κB binding sites (17, 28) (Fig. 3). Furthermore, several studies have demonstrated that this motif is important for Bcl-xL expression induced by CD40 stimulation in lymphocytes (27, 31). Thus, we analyzed whether NF-κB also contributes to Bcl-xL expression in TPO signaling by EMSA. The oligonucleotides containing this motif were used as probes (27). TPO treatment significantly enhanced the binding of a complex to this probe (Fig. 7A). A supershift study revealed that antibodies against p50 and c-Rel reduced the density of the complex, indicating that the complex contained p50 and c-Rel subunits of NF-κB (Fig. 7B). We also confirmed that p50 and c-Rel subunits of NF-κB are actually expressed in normal megakaryocytic cells, as in UT-7/TPO cells (Fig. 7C).

**PI 3-Kinase Is Required for Both NF-κB Activation and Bcl-xL Expression by TPO**—Because several cytokines activate NF-κB via PI 3-kinase (32, 33), we investigated whether TPO-induced NF-κB activation was also mediated by PI 3-kinase. For this purpose, we used a specific PI 3-kinase inhibitor, LY294002, and performed EMSA to detect NF-κB activity. As shown in Fig. 8A, LY294002 effectively inhibited the TPO-induced NF-κB activation at 20 μM. Concomitantly, LY294002 reduced Bcl-xL expression and induced apoptosis in a dose-dependent manner in UT-7/TPO cells (Fig. 8, B and C). However, it required a higher concentration of LY294002 to reduce the Bcl-xL expression level, compared with that required for inhibition of NF-κB (20 μM versus 100 μM). The Bcl-2 expression level was not altered after LY294002 treatment.
PI 3-Kinase Inhibitor LY294002 Enhanced TPO-induced DNA Binding of Stat5—As described above, PI 3-kinase inhibitor LY294002 reduced the TPO-induced NF-κB activation and protein level of Bcl-xL. Subsequently, LY294002 induced apoptosis in UT-7/TPO cells, although it required a higher concentration compared with that required for the suppression of NF-κB activation. These results prompted us to analyze the effects of LY294002 on Stat5 activation, another important transcription factor to regulate Bcl-xL gene expression as described above. As shown in Fig. 9A, LY294002 clearly enhanced the DNA binding activity of Stat5 without affecting Jak2 activation and Stat5 phosphorylation, we hypothesized that LY294002 may alter the subcellular distribution of Stat5. To confirm this notion, we prepared nuclear extracts for Western blotting analysis with anti-Stat5 antibody. As shown in Fig. 9C, the LY294002 treatment resulted in Stat5 accumulation in the nuclear fraction in a dose-dependent fashion. Furthermore, we analyzed the subcellular distribution of Stat5 using confocal microscopy (Fig. 10, A and B). After 24-h deprivation of TPO, Stat5 was mainly distributed in the cytoplasm in both untreated and LY-294002-treated UT-7/TPO cells. TPO induced the nuclear localization of Stat5 showing a punctate pattern of nuclear staining as described previously (34, 35). Three hours after TPO treatment, the Stat5 proteins moved to the cytoplasm in the control cells. Interestingly, when the cells were pretreated with LY294002,
Stat5 remained in the nucleus 3 h after the TPO stimulation. Twelve hours later, Stat5 moved to the cytoplasm in LY294002-treated cells as in the control cells. Taken together, these results indicate that PI 3-kinase regulates the subcellular localization of Stat5. In addition, LY294002-induced enhancement of Stat5 activity might compensate for down-regulation of the NF-κB activity, leading to maintenance of the Bcl-xL expression level.

FIG. 7. NF-κB activation after TPO treatment. A, enhanced NF-κB activity by TPO treatment. After 24 h of starvation, UT-7/TPO cells were stimulated with TPO for 3 h and nuclear extracts were prepared for EMSA. Oligonucleotides containing a putative NF-κB-binding site on the Bcl-xL gene promoter were used as probes. B, involvement of p50 and c-Rel subunits of NF-κB in TPO signaling. Five micrograms of the nuclear extracts was incubated for 15 min at room temperature with rabbit serum (lane 1) or antibodies against p50 (lane 2), p65 (lane 3), or c- Rel (lane 4). Then, EMSA was performed on the extracts using 32P-labeled oligonucleotides containing NF-κB binding site on the Bcl-xL gene promoter. C, expression of p50 and c-Rel subunits of NF-κB in normal megakaryocytic cells and UT-7/TPO cells. Total cell lysates from normal megakaryocytic cells or UT-7/TPO cells were loaded on SDS-PAGE and immunoblotted with anti-p50 or anti-c-Rel antibody.

FIG. 8. Inhibition of PI 3-kinase causes down-regulation of Bcl-xL and induces apoptosis in UT-7/TPO cells. A, effect of LY294002 on NF-κB activity. After 24-h deprivation, UT-7/TPO cells were incubated with the indicated concentrations of LY294002 for 1 h and subsequently stimulated with TPO for 3 h. The cells were then harvested, and EMSA was performed using oligonucleotide with consensus NF-κB-binding region on the Bcl-xL gene promoter. B, effect of Bcl-xL expression by LY294002 treatment. UT-7/TPO cells were cultured with LY294002 for 24 h and then total cell lysates were prepared for Western blotting analysis with anti-Bcl-xL or Bcl-2 antibody. C, effect of LY294002 on cell survival of UT-7/TPO cells. UT-7/TPO cells were cultured with the indicated concentrations of LY294002 for 24 h. The cells were then stained with anti-annexin V antibody and propidium iodine, and the ratio of apoptotic cells was analyzed by flow cytometry.
A fraction was then analyzed by Western blotting using anti-Stat5 antibody. The membranes were reprobed with anti-Jak-2 (upper panel) and anti-phosphotyrosyl Jak2 (lower panel) antibody. The membranes were then analyzed by Western blotting using anti-Stat5 antibody.

**FIG. 9.** Inhibition of PI 3-kinase enhanced DNA binding activity of Stat5. A, enhanced DNA binding activity of Stat5 by LY294002 treatment. UT-7/TPO cells were deprived of TPO for 24 h and then treated with various concentrations of LY294002 for 1 h. The cells were then stimulated with TPO (100 ng/ml) for the indicated periods and harvested for the preparation of nuclear fractions. Stat5 activation was analyzed by EMSA with β-casein promoter probe. B, effect of LY294002 on tyrosine phosphorylation of Jak2 and Stat5. After 24-h starvation, UT-7/TPO cells were cultured with LY294002 for 1 h and then stimulated with TPO (100 ng/ml) for 7 min. Total cell lysates were prepared and resolved by SDS-PAGE. The membranes were reprobed with anti-Jak-2 (upper panel) and anti-Stat5 (lower panel). C, accumulation of Stat5 in the nuclear fraction by LY294002 treatment. After 24 h of starvation, UT-7/TPO cells were cultured with LY294002 for 1 h and then stimulated with TPO (100 ng/ml) for 15 min or 3 h. Total cell lysates were prepared and resolved by SDS-PAGE. The expression level of Stat5 in the nuclear fraction was then analyzed by Western blotting using anti-Stat5 antibody.

**DISCUSSION**

In this study, we focused our attention on elucidating the precise mechanisms through which TPO maintains the survival of megakaryocytic progenitor cells. We showed that the Bcl-xL protein, an important member of the Bcl-2 family, is involved in TPO-dependent cell survival. We also showed that two distinct signal transduction pathways, Jak2-Stat5 and PI 3-kinase activation pathways, are required for TPO-induced transcriptional regulation of the Bcl-xL gene expression. Finally, we showed that subcellular localization of Stat5 is in part dependent on PI 3-kinase activity.

Bcl-xL works as an essential anti-apoptotic factor in various types of cells including hematopoietic cells (7). It was previously reported that primitive hematopoietic progenitor cells express Bcl-xL and that Bcl-xL is required for the long-term survival of the hematopoietic stem cells (11, 12). In addition, it was reported that Bcl-xL is required for the differentiation and maturation of committed hematopoietic progenitor cells. For example, Bcl-xL has crucial roles in the development of erythroid lineage cells (13, 14, 36). Mononuclear differentiation of myeloid lineage progenitor cells also requires Bcl-xL expression (15). Furthermore, Bcl-xL is also expressed in normal megakaryocytic progenitor cells (25). In this study, we showed that Bcl-xL is inducibly expressed by TPO in TPO-responsive human leukemic cell line UT-7/TPO and normal human megakaryocytes. We also showed that Bcl-2, another important anti-apoptotic Bcl-2 family member (7), is detectable in UT-7/TPO cells and normal megakaryocytic cells. However, TPO deprivation did not affect Bcl-2 expression. Furthermore, TPO stimulation did not induce Bcl-2 expression. Although we could not completely exclude the possibility that Bcl-2 has some anti-apoptotic function in megakaryocytic cells, our data strongly suggested that Bcl-xL expression is a prerequisite for cell survival in megakaryocytic lineage cells.

The expression of Bcl-xL in hematopoietic cells is mainly regulated by hematopoietic cytokines at the transcriptional level (17). Consistent with this notion, the regulatory region of the Bcl-x gene contains several cytokine-responsive elements, including Stat-binding sites and NF-kB binding sites (17, 28). Among them, Stat proteins are a unique family of proteins that dually function as signal transducers and transcription factors. It is well-known that Stat proteins are activated by a variety of cytokines and control the growth, survival, or differentiation of hematopoietic cells (37, 38). Several studies have reported that Bcl-xL is one of the most important target genes of Stat proteins; however, the activation pattern of Stat proteins involved in Bcl-xL gene control is different in each cytokine. In EPO-dependent murine cell line HCD-57 cells, Stat5 regulates the Bcl-xL gene in response to EPO (18). Stat5 is also involved in IL-3-induced Bcl-xL gene expression (19). In contrast, LIF up-regulates Bcl-xL gene expression through Stat1 activation in cardiocytes (39). Furthermore, Stat 3 plays a crucial role in IL-6-dependent Bcl-xL gene expression in myeloma cell line U-266 (40). Stat6 also has the ability to regulate the Bcl-xL gene in IL-15 signaling (20). TPO activates Stat3 and Stat5 in normal megakaryocytes (23). In addition, we previously reported that TPO activates Stat3 and Stat5 in UT-7/TPO cells (41). In this study, we found that Stat5 but not Stat3 binds to the putative Stat-binding site on the Bcl-x gene promoter. Furthermore, a dominant negative mutant of Stat5 but not Stat3 clearly inhibited the TPO-induced Bcl-x gene promoter activity. These results indicate that Stat5 has the capacity to control the Bcl-x gene in TPO signaling. Previously, we showed that Stat3 activation is correlated with TPO-induced cellular proliferation (41). Transgenic mice with a dominant negative Stat3 using the GATA-1 gene promoter regulatory region revealed a delayed recovery of the number of megakaryocytes and of platelet production after 5-fluorouracil-induced thrombocytopenia. However, we did not observe any significant increase in the ratio of apoptotic cells in the megakaryocytic lineage cells of these mice. Together with these observations, it is hypothesized that Stat3 and Stat5 have distinct functions in TPO signaling; Stat3 regulates cell growth and Stat5 works as an anti-apoptotic effector. It would be attractive to analyze the effects of genetic elimination of Stat5a and/or Stat5b on the development of megakaryocytic lineage cells.
megakaryopoiesis, especially on the survival of megakaryocytic cells.

In addition to Stat proteins, NF-κB plays an important role in gene regulation of Bcl-xL in many types of cells. There are some putative NF-κB-binding sites on the regulatory region of the Bcl-x gene as illustrated in Fig. 3 (17, 28). Lee et al. (27) reported that the NF-κB-binding sites (III) 77 bp and 62 bp upstream of the transcription start sites are required for CD40-mediated Bcl-xL expression in B cells. In contrast, Glasgo et al. (42) reported that other NF-κB-binding sites are located in the region of about 847 bp (II) and 967 bp (I) upstream of the transcription start sites, and that this region is crucial for Bcl-xL gene expression in pheochromocytoma cell line PC12 cells. In this study, we showed that deletion of NF-κB binding sites I and II did not affect the activity of the Bcl-x gene promoter (Fig. 3). However, deletion of the region containing NF-κB binding sites III and Stat-binding sites reduced the Bcl-xL promoter activity. Based on these findings, we concluded that proximal (III) but not distal NF-κB binding sites (I and II) are essential for TPO-induced Bcl-xL expression. Indeed, we found that TPO enhanced the binding of NF-κB complex on this proximal NF-κB binding site I. The NF-κB family of proteins includes p50, p52, p65, c-Rel, and Rel-B (43, 44). Among them, we showed that p50 and c-Rel subunits bind to the Bcl-xL gene promoter. This is consistent with a previous report that p50 and c-Rel can bind to the NF-κB binding sites on the Bcl-xL gene promoter (45). In addition, we showed that normal megakaryocytes express these two subunits (Fig. 7C). p50 and c-Rel are also expressed in CD34-positive hematopoietic cells and are required for the survival of these cells (46). In contrast, committed erythroid progenitors (BFU-E) mainly express p50, p52, and p65 (47). Therefore, the combination of NF-κB subunits expressed in hematopoietic cells depends on cell lineage.

In UT-7/TPO cells, LY294002 reduced the TPO-induced DNA binding activity of NF-κB, indicating that TPO-induced activation of NF-κB is dependent on PI 3-kinase activity. Recent studies have revealed that PI 3-kinase induced DNA binding activity of NF-κB through Akt activation and subsequent IκB phosphorylation (32, 33). We previously showed that TPO activated Akt in a PI 3-kinase-dependent manner (29). Taken together, the DNA binding activity of NF-κB may be enhanced through TPO-induced PI 3-kinase-Akt activation.

It is noteworthy that there was a large discrepancy between the Bcl-xL level and the DNA binding activity of NF-κB. The expression level of Bcl-xL proteins was not down-regulated by treatment with 50 μM LY294002, at which dosage the DNA binding activity of NF-κB was drastically suppressed in UT-7/TPO cells. A possible explanation for this discrepancy is that decreased NF-κB activity was compensated for Stat5, resulting in the maintenance of the Bcl-xL level. Surprisingly, LY294002 clearly enhanced the DNA binding activity of Stat5. This finding suggested that PI 3-kinase is one of the important upstream modulators of Stat proteins. However, the LY294002 treatment did not affect the Jak2 activation or tyrosine phos-
phorylation of Stat5. Interestingly, we found that nuclear localization of Stat5 was enhanced by LY294002 treatment. The nuclear transport mechanism of Stat5 has not been well described, and it is unclear how PI 3-kinase regulates Stat5 distribution. Recent work by Ginger et al. (48) may provide an explanation for this mechanism. They demonstrated that Gsk-A, a Dictyostelium homolog of glycogen synthase kinase-3 (GSK-3) regulates the nuclear translocation of Dictyostelium Stat proteins, Dd-StatA; serine phosphorylation by GskA promoted the nuclear export of Dd-StatA. Importantly, mammalian Stat5B contains potential GSK-3 phosphorylation sites in the N-terminal region. Recently, we reported that TPO activates GSK-3 in a PI 3-kinase- and Akt-dependent manner in UT-7/TPO cells (29). Collectively, PI 3-kinase may regulate the function of Stat5 through the activation of GSK-3 and subsequent changes in subcellular localization of Stat5. If so, Stat5 can work as a rescue unit to maintain Bcl-xL under an emergency condition in which PI 3-kinase function is abrogated. We showed here that TPO supports the survival of megakaryocytic cells, in part mediated through the induction of the anti-apoptotic protein, Bcl-xL. In addition, we showed that Bcl-xL gene expression is mainly regulated by the Jak2-Stat5 and PI 3-kinase activation pathways in TPO signaling. Surprisingly, we found that the subcellular distribution of Stat5 is in part regulated by PI 3-kinase activity. This suggested that there is a possible cross-talk in Bcl-xL gene regulation between the Jak-Stat and PI 3-kinase activation pathways. Although the concept is speculative at present, the cross-talk between them may contribute to the redundancy of TPO signaling to support the survival of megakaryocytic lineage cells.

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