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Thrombopoietin Induces Tyrosine Phosphorylation of a Common β Subunit of GM-CSF Receptor and Its Association with Stat5 in TF-1/TPO Cells

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TF-1/TPO cells are derived from an erythroleukemia cell line, TF-1, and are absolutely dependent on either TPO or granulocyte-macrophage colony-stimulating factor (GM-CSF)/interleukin-3 (IL3) for their continuous growth and survival. To gain insight into the molecular basis of hemopoietic activities shared by TPO and GM-CSF/IL3 in TF-1/TPO cells, we studied the cross-talk between signal transduction pathways elicited by these cytokines. Stimulation of TF-1/TPO cells with TPO resulted in tyrosine phosphorylation of the TPO receptor (c-Mpl) as well as the common β subunit (βc) of GM-CSF/IL3 receptor complex. GM-CSF, however, induced tyrosine phosphorylation of βc but not c-Mpl. TPO-induced tyrosine phosphorylation of βc was time- and dose-dependent. We next examined whether or not TPO-induced tyrosine phosphorylation of βc led to recruitment of SH2-containing molecules such as Stat5 and Shc. While GM-CSF caused association of Stat5 and Shc with βc, TPO caused association of Stat5, but not Shc, with βc, suggesting that TPO and GM-CSF may not induce phosphorylation of the same sets of tyrosine residues in βc. These results suggest that activation of c-Mpl affects the signaling pathway of GM-CSF/IL3 but not *vice versa*.

Thrombopoietin (TPO) is known to play a crucial role in the growth and development of megakaryocytic progenitor cells as well as the production of platelets (1-4). It has also been reported that TPO expands not only megakaryocytic but also erythroid and granulocyte-macrophage progenitor cells in normal and myelosuppressed mise, suggesting that TPO may be active on a rather wide variety of hemopoietic cells (5). Indeed it stimulates the growth of a significant portion of primary acute myelogenous leukemia (AML) cells (6). The TPO receptor, also called c-Mpl, is a member of cytokine receptor superfamily and forms homodimer upon ligand binding (7, 8), leading to activation of JAK2 and TYK2 kinases and then tyrosine phosphorylation of various SH2-containing signaling molecules including Shc, Stat3 and Stat5 (9-18).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL3) have overlapping biological effects on the proliferation and differentiation of immature myeloid progenitor cells as well as the functional activation of more mature myeloid cells. The functional receptors for GM-CSF, IL3 and interleukin-5 (IL5) consist of a specific component (α subunit) and a shared one (common β subunit; βc) (19, 20). The latter is mainly responsible for signal transduction, and becomes phosphorylated on several tyrosine residues by JAK2 kinase when stimulated with ligands. Then βc binds a number of cytoplasmic proteins through the interaction between phosphotyrosine and SH2 motif (19-22). Most of these proteins are common in recruitment in response to GM-CSF/IL3/IL5, granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO) and TPO.

Recently some reports have documented that EPO and G-CSF induced unidirectional cross-phosphorylation of βc or mouse IL3 receptor β subunit (βIL3) via their cognate receptors (23-25). It was also reported that stem cell factor (SCF) caused association of its tyrosine kinase receptor, c-Kit, with EPO receptor, and that SCF indirectly activated EPO signaling pathway (26-28). In the present study, we studied the cross-talk between signal transduction pathways elicited by TPO and GM-CSF in TF-1/TPO cells, which are absolutely dependent on either TPO or GM-CSF/IL3 for their continuous growth and survival.

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MATERIALS METHODS

Reagents. Recombinant human TPO, recombinant human GM-CSF and rabbit polyclonal antibody against human c-Mpl were provided by Kirin Brewery Co Ltd (Gumma, Japan). Recombinant anti-phosphotyrosine (RC20; catalog number E120H) and polyclonal antibody against Shc (catalog number S14630) were purchased from Transduction Laboratories (Lexington, KY). Mouse monoclonal antibody (S-16; catalog number sc-457) and rabbit polyclonal antibody (C-20; catalog number sc-675) against common β subunit of GM-CSF, IL3, and IL5 receptors, rabbit polyclonal antibody against JAK2 (C-20; catalog number sc-294) and rabbit polyclonal antibody against Stat5b (C-17; catalog number sc-835) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell cultures. TF-1/TPO cells were cultured in Iscove's modified Dulbecco's medium (IMDM; GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and 5 ng/ml of rhTPO at 37°C.

Immunoprecipitation and immunoblot analysis. TF-1/TPO cells were starved for cytokines overnight. The cells were then adjusted at 1×10^7 cells/ml and stimulated with TPO, GM-CSF or medium only for the indicated periods at 37°C. Cells were then washed twice with cold PBS and lysed for 5 min at 4 °C with 1% NP-40 containing 50 mM HEPES, 150 mM NaCl, 1 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 500 IU/ml aprotinin, and 10% glycerol. Cell lysates were collected following centrifugation at 15,000 rpm for 10 min, incubated with the primary antibody for 2 hrs at 4°C, added to 20 μl protein A sepharose beads slurry (50%), and incubated for further 1 hr at 4°C. Beads were washed three times with a washing buffer and boiled in 1 × Laemmli's sample buffer before electrophoresis. Immunoprecipitates were analyzed on 7.5% polyacrylamide-SDS gels, and then transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Membranes were blocked by incubation with Tris-buffered saline containing 0.1% TritonX-100 and 1% BSA, and immunoblot analysis was performed by employing the method of enhanced chemiluminescence (Amersham). To confirm proper loading of proteins in each lane, membranes were stripped in 2% SDS, 62.5 mM Tris-HCl (pH 6.8), and 100 mM 2-mercaptoethanol at 50°C for 30 min, blocked, and re-probed with antibodies as indicated.

RESULTS

We have established a TPO-dependent subclone, designated TF-1/TPO, from human erythroleukemia-derived TF-1 cells which exhibit only a weak mitogenic response to TPO. TF-1/TPO cells respond to both GM-CSF and TPO in a similar dose-dependent manner (to be submitted elsewhere). To gain insight into the molecular basis of hemopoietic activities shared by TPO and GM-CSF in this cell line, we examined the cross-talk between the signal transduction pathways of these two cytokines.

Cytokine-starved TF-1/TPO cells were stimulated with 100 ng/ml of GM-CSF or TPO for 5 min and were examined for induction of tyrosine phosphorylation of βc. βc was precipitated from the cell lysates with specific antibodies (S-16), followed by immunoblot analysis with anti-phosphotyrosine antibodies. βc was markedly phosphorylated on tyrosine residues in response to GM-CSF as expected (Fig.1A). When stimulated with TPO, weak but reproducible phosphorylation of βc was also observed (Fig.1A). Then, tyrosine-phosphorylation

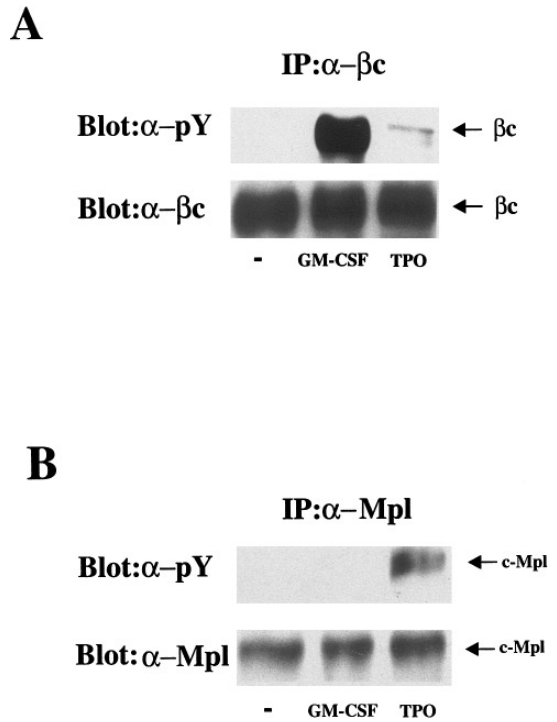

FIG. 1. (A) Tyrosine phosphorylation of common β subunit by TPO stimulation in TF-1/TPO cells. TF-1/TPO cells were starved of cytokines overnight. The cells were then adjusted at 1×10^7 cells/ml and stimulated with TPO (100 ng/ml), GM-CSF (100 ng/ml) or medium only for 5 min at 37 °C. The cells were lysed at 4 °C in lysis buffer and then immunoprecipitated with βc antibody (S-16). Immunoprecipitates were separated on SDS 7.5% polyacrylamide gels and analyzed by immunoblotting with anti-phosphotyrosine antibody RC20. To confirm that a similar amount of βc was loaded in each lane, membranes were stripped and reprobed with βc antibody (C-20). (B) Tyrosine phosphorylation of c-Mpl in TF-1/TPO cells. TF-1/TPO cells were starved of cytokines overnight. The cells were then adjusted at 1×10^7 cells/ml and stimulated with TPO (100 ng/ml), GM-CSF (100 ng/ml) or medium only for 5 min at 37 °C. The cells were lysed at 4 °C in lysis buffer and then immunoprecipitated with c-Mpl antibody. Immunoprecipitates were separated on SDS 7.5% polyacrylamide gels and analyzed by immunoblotting with anti-phosphotyrosine antibody RC20. To confirm a similar amount of c-Mpl was loaded in each lane, membranes were stripped and reprobed with anti c-Mpl antibody.

of c-Mpl in response to these cytokines was tested. TPO but not GM-CSF induced tyrosine-phosphorylation of c-Mpl (Fig.1B).

Next, TF-1/TPO cells were stimulated with various concentrations of TPO for 5 min. As shown in Fig.2A, tyrosine phosphorylation of βc could be detected as low as at 1 ng/ml TPO, reached the maximal intensity at 10 ng/ml, and curiously rather decreased at 100 ng/ml. Subsequent studies were therefore performed at 10 ng/ml TPO. Tyrosine-phosphorylation of βc through c-Mpl was also time dependent. It was observed as fast as 1 min after 10 ng/ml TPO stimulation, reached maximal intensity at 5 min, and then declined at 20 min (Fig.2B).

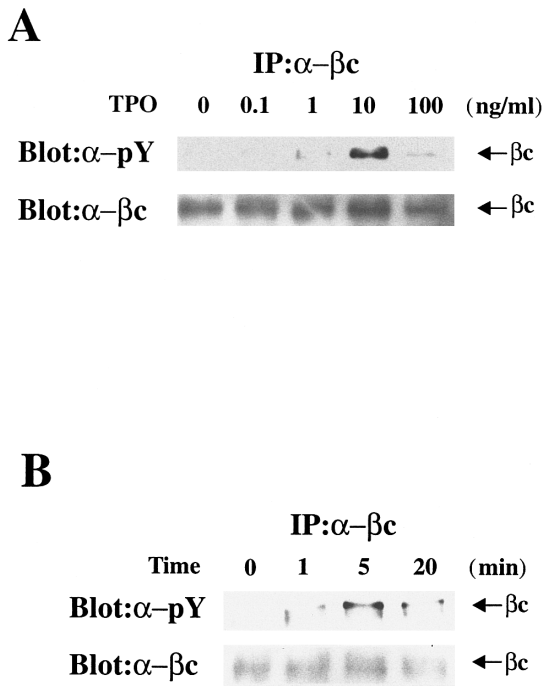

FIG. 2. (A) Dose response of βc tyrosine phosphorylation by TPO stimulation in TF-1/TPO cells. TF-1/TPO cells were stimulated for 5 min with the indicated concentration of TPO. The cells were lysed at 4 °C in lysis buffer and then immunoprecipitated with βc antibody (S-16). Immunoprecipitates were separated on SDS 7.5% polyacrylamide gels and analyzed by immunoblotting with anti-phosphotyrosine antibody RC20. To confirm that a similar amount of βc was loaded in each lane, membranes were stripped and reprobed with βc antibody (C-20). The experiments were repeated two times with similar results. (B) Time course of βc tyrosine phosphorylation by TPO stimulation in TF-1/TPO cells. TF-1/TPO cells were stimulated with Tpo (10 ng/ml) for 0 to 20 min. The cells were lysed at 4 °C in lysis buffer and then immunoprecipitated with βc antibody (S-16). Immunoprecipitates were separated on SDS 7.5% polyacrylamide gels and analyzed by immunoblotting with anti-phosphotyrosine antibody RC20. To confirm that a similar amount of βc was loaded in each lane, membranes were stripped and reprobed with βc antibody (C-20). The experiments were repeated two times with similar results.

We next examined whether or not TPO-induced tyrosine phosphorylation of βc led to recruitment of SH2-containing molecules such as Stat5 and Shc, which had been reported to be associated with βc upon GM-CSF binding. Cytokine-starved TF-1/TPO cells were stimulated with 10 ng/ml of TPO for 5 min, and examined for association of JAK2, Stat5, or Shc with βc. βc was precipitated from the cell lysates with anti-βc antibodies (S-16), followed by immunoblot analysis with anti-JAK2, Stat5, or Shc antibodies. As shown in Fig.3A, the association of JAK2 with βc was observed in unstimulated TF-1/TPO cells, and stimulation with TPO did not significantly enhance this association. In contrast, the association between Stat5 and βc was not detected in unstimulated TF-1/TPO cells and TPO induced the binding of Stat5 to βc (Fig.3A). The association of Stat5 with βc in TPO-stimulated TF-1/TPO cells was further demonstrated when the cell lysate was immunoprecipitated with the anti-Stat5 antibody, followed by immunoblotting with anti-βc antibody (Fig.3B). However, the association of βc with JAK2 was not demonstrated by immunoprecipitation with the anti-JAK2 antibody, followed by immunoblotting with the anti-βc antibody (data not shown). This result was apparently inconsistent with the result of coimmunoprecipitation of JAK2 and βc shown in Fig.3A. Although the reason is not clear at present, the possibilities may exist such as poor efficiency of immunoprecipitation with the anti-JAK2 antibody. The association of βc with Shc in TF-1/TPO cells was not detected with or without TPO stimulation when immunoprecipitation was performed with the anti-βc antibody or the anti-Shc antibody (data not shown).

DISCUSSION

Many investigators have reported that protein-tyrosine phosphorylation plays a critical role in the signal transduction through ligand binding to the cytokine receptor superfamily, including c-Mpl (9-18). TPO affects not only megakaryocytic lineage progenitor but also erythroid, granulocyte-macrophage lineage progenitor (5). Recently unidirectional cross-phosphorylation of the β subunit of the GM-CSF or IL3 receptor by EPO (23,24) or G-CSF (25) stimulation has reported. c-Mpl and βc belong to the cytokine receptor superfamily, which are defined by the regions with similarity in their exoplasmic domains and lacking kinase sequences in their cytoplasmic domains. Both TPO and GM-CSF induce tyrosine phosphorylation of JAK2 and Stat5. We established a TPO-dependent subclone, TF-1/TPO, from GM-CSF or IL3 dependent human leukemia cell line TF-1, and used this new cell line for analysis of cytokine receptor interaction after TPO stimulation.

In this report we found tyrosine phosphorylation of βc after TPO stimulation. Tyrosine phosphorylation of βc by TPO stimulation raises at least two possibilities. One is that TPO may activate one or more tyrosine kinases, such as JAK2, after binding to its receptor, c-Mpl, then the tyrosine kinase(s) induces tyrosine phosphorylation of βc. It is thought that βc is tyrosine phosphorylated by JAK2 after ligand binding (21). We did not examine whether JAK2 or TYK2 induced tyrosine phosphorylation of βc in this system. Another possibility is that βc may act as a subunit of c-Mpl. Although, like the receptor for EPO (30) and G-CSF (31), c-Mpl is believed to be activated through homodimerization (32), the existence of an additional unidentified subunit can not be fully ruled out.

We also found binding of Stat5 to βc after TPO stimulation. This finding is similar to the report that EPO induces tyrosine phosphorylation of β_{IL3} and recruitment of Stat5 to possible Stat5-docking sites of β_{IL3} in

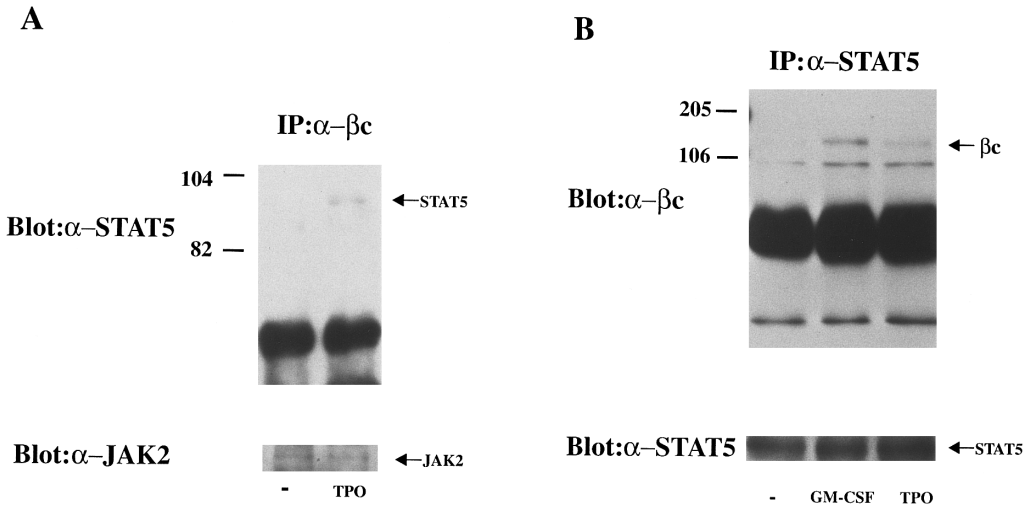

FIG. 3. (A) Co-immunoprecipitation of JAK2 and Stat5 with βc. TF-1/TPO cells were starved of cytokines overnight. The cells were then adjusted at 1×10^7 cells/ml and stimulated with TPO (10 ng/ml) or medium only for 5 min at 37 °C. The cells were lysed at 4 °C in lysis buffer and then immunoprecipitated with βc antibody (S-16). Immunoprecipitates were separated on SDS 7.5% polyacrylamide gels and analyzed by immunoblotting with anti-JAK2 or Stat5 antibody. The size markers are indicated and given in kD. (B) Co-immunoprecipitation of βc with Stat5. TF-1/TPO cells were starved of cytokines overnight. The cells were then adjusted at 1×10^7 cells/ml and stimulated with TPO (10 ng/ml), GM-CSF (100 ng/ml), or medium only for 5 min at 37 °C. The cells were lysed at 4 °C in lysis buffer and then immunoprecipitated with Stat5 antibody. Immunoprecipitates were separated on SDS 7.5% polyacrylamide gels and analyzed by immunoblotting with βc antibody (C-20). To confirm that a similar amount of Stat5 was loaded in each lane, membranes were stripped and reprobed with Stat5 antibody. The size markers are indicated and given in kD.

a murine model (24). However, another SH2-containing signaling molecule, Shc, which is thought to be involved in activation of the Ras-MAP kinase pathway, was not recruited to tyrosine-phosphorylated βc after TPO stimulation. These findings suggest that TPO induces phosphorylation of the tyrosine residues specific for Stat5 binding on βc. It is necessary to elucidate which tyrosine kinases can induce tyrosine phosphorylation of βc after TPO stimulation and to study biological significance of tyrosine phosphorylation of βc after TPO stimulation in TF-1/TPO cells.

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