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THROMBOPOIETIN INDUCES TYROSINE PHOSPHORYLATION AND ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASES IN A HUMAN THROMBOPOIETIN-DEPENDENT CELL LINE

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Summary: Thrombopoietin (TPO) is a cytokine which can support the proliferation and differentiation of megakaryocyte progenitor cells, and the maturation of megakaryocytes. We show here that mitogen-activated protein (MAP) kinases, Erk1 and Erk2, are involved in TPO signal transduction in the human TPO-dependent megakaryocytic cell line, UT-7/TPO. TPO induced tyrosine phosphorylation of Erk1 and Erk2 proteins in a dose and time-dependent manner. Moreover, the activation of MAP kinases was actually induced by TPO. These results suggest that MAP kinase activation is involved in the signalling pathway of TPO, as it is for other cytokines, one of which is erythropoietin.

Thrombopoietin (TPO), c-Mpl ligand, is a newly cloned cytokine which acts by regulating proliferation and differentiation of megakaryocytes, and the level of circulating platelets. It is composed of an N-terminal domain homologous to erythropoietin (EPO) and a glycosylated carboxyl domain with an unknown function. The TPO receptor, encoded by the proto-oncogene *c-mpl*, is a member of the cytokine receptor superfamily. Its expression appears to be restricted to human CD34-positive hematopoietic cells, megakaryocytic lineage cells, and circulating platelets (1, 2).

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Despite recent work on c-Mpl and its ligand, its postreceptor signalling pathway is still not fully understood. In general, ligand-binding to the receptor leads to aggregation of several subunits, and a receptor complex is formed. This complex activates intracellular tyrosine kinases which are responsible for phosphorylation of the receptor itself and a number of transducing molecules (3). Although, like other members of the cytokine receptor superfamily, the TPO receptor has no intrinsic tyrosine kinase activity, TPO induces tyrosine phosphorylation of the JAK2 tyrosine kinase, VAV, p85 subunit of phosphatidylinositol 3-kinase (PI3-K p85) and Shc proteins in several TPO-dependent cell lines (4-8) and platelets (9). These proteins are also tyrosine phosphorylated by erythropoietin (EPO), interleukin-3 (IL-3), or granulocyte-macrophage colony-stimulating factor (GM-CSF), suggesting that considerable overlap exists between signalling pathways activated by different receptors (3).

MAP kinases, also called extracellular signal-regulated kinases (Erk) are known to lie at the convergence of various extracellular ligand-mediated signalling pathways and they are associated with cell growth and differentiation. Several growth factors and cytokines including IL-3, GM-CSF, and EPO have been shown to activate the Ras-MAP kinase pathway (10-13). These findings prompted us to examine whether or not TPO also induces tyrosine phosphorylation and activation of MAP kinases.

Recently, we have established a novel human leukemia cell line, UT-7/TPO (14). UT-7/TPO cells are TPO-dependent for growth and survival, and they have also mature megakaryocytic features. In this study, we found that TPO induces tyrosine phosphorylation and activation of MAP kinases in UT-7/TPO cells. This suggested that MAP kinase activation is also involved in the TPO signalling pathway. Thus, MAP kinases may play an important role in megakaryopoiesis.

MATERIALS AND METHODS

Cell culture: The UT-7 cell line was established from bone marrow cells of a patient with acute megakaryocytic leukemia (15). UT-7/EPO (16), a subclone of UT-7, were grown in Iscove's modified Dulbecco's medium (IMDM), supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) and 1 U EPO/ml. UT-7/TPO is a recently established TPO-dependent megakaryocytic cell line derived from UT-7/GM (14), and was maintained with IMDM containing 10% FCS and 10 ng/ml of human recombinant TPO (a gift from Kirin Brewery Co., Pharmaceutical Research Laboratory, Gunma, Japan).

Reagents: Anti-rat MAP kinase (erk1-CT) polyclonal antibody, anti-MAP kinase (erk2) monoclonal antibody and anti-phosphotyrosine (anti-Ptyr) monoclonal antibody were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit anti-mouse IgG, goat anti-rabbit IgG conjugated with horseradish peroxidase and enhanced chemiluminescence (ECL) Western blotting reagents were purchased from Amersham (Arlington Heights, IL). Protein G sepharose was purchased from Pharmacia (Uppsala, Sweden).

Preparation of cell lysates, immunoprecipitation and Western blotting: UT-7/EPO and UT-7/TPO cells were starved of growth factor for 24hrs. After cytokine stimulation at 37°C for 10 minutes, or following a time course, cells were washed twice in cold PBS and lysed in a buffer containing 20 mM Tris (pH 7.4), 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM phenylmethyl-sulfonilfluoride (PMSF), 15 μg/ml aprotinin and 2 mM sodium orthovanadate. This was followed by a 20 min incubation on ice. The insoluble particles were precipitated by centrifugation at 12,000g for 10 min. The supernatant was saved and immunoprecipitated with monoclonal antibody (4G10) coated protein G sepharose for 4h at 4°C in an Eppendorf shaker. Immunoprecipitates were collected by brief centrifugation, washed five times with 1ml of lysis buffer, and boiled for 5 min in SDS-PAGE sample buffer. After brief centrifugation, the supernatant was resolved by SDS-PAGE, then electroblotted onto nitrocellulose membranes. The blots were blocked with 5% low fat milk powder in TBS containing Tween 20 (1:2,000) (TBS-T) for 1h at room temperature. After washing with TBS-T, they were incubated with an appropriate concentration of primary antibody for 1h. After washing with TBS-T again, the blots were probed with 1:1,000 dilution of goat anti-rabbit, or rabbit anti-mouse, horseradish peroxidase-conjugated secondary antibody for 1h. Following the third wash, the blots were incubated with an enhanced chemiluminescence substrate (Amersham) for 1 min, and exposed to film to visualize immunoreactive bands.

Assay of MAP kinase activity: MAP kinase activity in the cells was assayed as follows. The cells were starved for 24 hrs. After adjusting the number of cells to $1x10^6$ cells/sample and washing twice with physiolosical saline solution, the cells were exposed to 10 U/ml of EPO or 100 ng/ml of TPO at 37°C for a certain time. The reaction was stopped by addition of 0.4 ml of ice-cold extraction solution (20 mM Tris HCL, 6 mM EGTA, 6 mM $MgCl_2$, 0.1 mM sodium fluoride, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 20 μg/ml aprotinin, 1 mM PMSF, pH 7.4) on dry ice. The cell extracts were centrifuged at 1,500 g for 1 min. The supernatant was incubated with 6 mM of a synthetic peptide (sequence APRTPGGRR, amino acids 95-98 of bovine myelin basic protein (MBP) with the Thr-97 phosphorylation site for MAP kinase) and reaction mixture containing 75 mM glycero-phosphoric acid, 2 mM dithiothreitol, 6 mM $MgCl_2$, and 50 μM ^{32}P-γATP (specific activity; 10 Ci/mmol, New England Nuclear, Wilmington, MA) for 10 min at 25°C. This synthetic peptide is more selective for MAP kinases than is MBP, as a substrate (17). The reaction products were placed on P-81 phosphocellulose papers (Whatman International Ltd., Maidstone, England) and washed in 20 ml of ice-cold 10 mM phosphoric acid three times. The radioactivity was counted by a liquid scintillation counter. Specific radioactivity was obtained by subtracting the radioactivity of the synthetic peptide-free reaction from the synthetic peptide-directed radioactivity. MAP kinase activity was represented as p moles of ATP incorporated per $1x10^6$ cells during 1 min.

RESULTS

TPO Stimulates Phosphorylation of Cellular Proteins Including Erk1 (44kDa) and Erk2 (42kDa). UT-7/TPO cells showed a rapid increase in tyrosine phosphorylation in

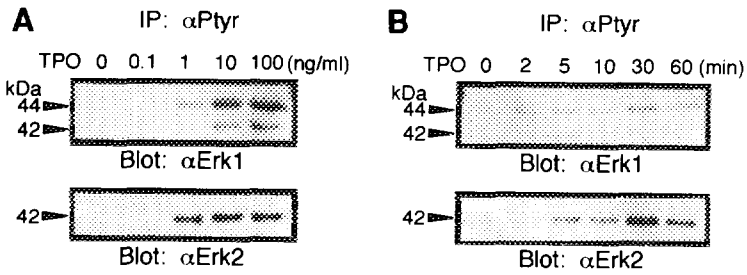

Figure 1. **Tyrosine phosphorylation of MAP kinases, Erk1 and Erk2, in response to TPO.** UT-7/TPO cells were deprived of growth factors for 24 hrs. After TPO-stimulation, the cells were lysed and phosphotyrosine proteins of the lysates were immunoprecipitated by a monoclonal antibody against phosphotyrosine (4G10), blotted onto nitrocellulose membrane, and probed with anti-Erk1 (upper panel) or anti-Erk2 antibody (lower panel). (A) Starved UT-7/TPO cells were exposed to TPO at the increasing concentrations (0-100 ng/ml) for 10 min and harvested. The arrows indicate the size of the tyrosine-phosphorylated Erk1 and Erk2 proteins. (B) Time course of TPO-induced tyrosine-phosphorylation of Erk1 and Erk2 proteins. Starved UT-7/TPO cells were exposed to 10 ng/ml of TPO for the times indicated and harvested.

response of TPO. The prominent tyrosyl phosphorylated proteins were the 145 kDa (PLC-γ1), 130 kDa (JAK2), 120 kDa (GTPase activating protein), 66 kDa, 52 kDa, and 46 kDa (Shc) proteins (data not shown; Ref. 14 and unpublished data). Less intense bands were observed at 40 to 50 kDa, which we think represents MAP kinases. Since suitable anti-Erk antibodies for immunoprecipitation were not available in our laboratory, whole cell lysates were precipitated with anti-phosphotyrosine monoclonal antibody (4G10), blotted onto nitrocellulose membrane, and probed with anti-Erk1 or anti-Erk2 antibody. UT-7/TPO cells were exposed to increasing concentrations of TPO (0.1-100 ng/ml) for 10 min. The tyrosine phosphorylated Erk1 (44 kDa) and Erk2 (42 kDa) proteins were detected at 1 ng/ml of TPO, and a plateau level was obtained at 10 ng/ml (Fig. 1A). The concentration of TPO required to induce tyrosine phosphorylation of these proteins was in the same range of concentration required to stimulate the proliferation of UT-7/TPO cells (data not shown). Moreover, by increasing the time of exposure to TPO (100 ng/ml) up to 60 min, we found that the tyrosine phosphorylation of Erk1 and Erk2 proteins appeared within 2 min, and peaked at 30 min, and had diminished by 60 min (Fig. 1B). Thus, TPO induced tyrosine phosphorylation of Erk1 and Erk2 proteins in a dose and time-dependent manner.

The MAP kinases are activated by TPO stimulation. To confirm the activation of MAP kinases, we measured MAP kinase-specific activities by using a ^{32}P-labeled synthetic

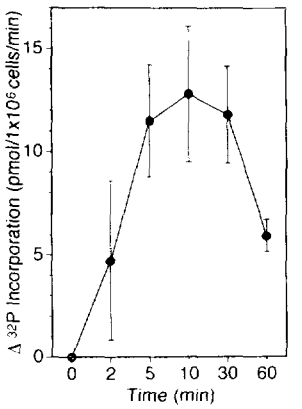

Figure 2. **Kinetics of TPO-induced MAP kinases activities.** UT-7/TPO cells were starved of growth factors for 24 hrs. Then, the cells were exposed to 100 ng/ml of TPO for the times indicated. The cell extracts were incubated with a synthetic peptide (APRTPGGRR) and reaction mixture containing ^{32}P-γATP. The radioactivity incorporated into the synthetic peptide was analyzed. MAP kinase activity was represented as p moles of ATP incorporated per 1x10^6 cells during 1 min. Δ^{32}P incorporation was calculated by subtracting the ^{32}P incorporation of untreated cells from that of TPO-treated cells. The experiment was repeated twice, and representative data are shown as mean ± SD (n=3).

peptide which is part of the myelin basic protein, as a substrate. The MAP kinase activity in UT-7/TPO cells increased within 2 min of TPO treatment (100 ng/ml), peaked after 10 min, and had diminished by 60 min as shown in Fig. 2.

The MAP kinases are also tyrosine phosphorylated and activated by EPO stimulation. There have been several reports about the activation of MAP kinases using murine cell lines transfected with EPO receptor cDNA (13,14), and human erythroleukemia cell line, TF-1 (12). To compare the intracellular signalling events by TPO and EPO in UT-7 cell lines, we also examined the Epo-induced tyrosine phosphorylation and activation of MAP kinase in UT-7/EPO, an EPO-dependent cell line with erythroid features (16). EPO induced tyrosine phosphorylation of Erk1 and Erk2 proteins in a dose and time-dependent manner in UT-7/EPO cells. The tyrosine phosphorylated Erk1 and Erk2 proteins were detected at 0.1 U/ml of EPO, and a plateau level was obtained at 1 U/ml (Fig. 3A). These concentrations were comparable to those stimulate the proliferation of UT-7/EPO cells (16). The tyrosine phosphorylation of these proteins appeared within 2 min, peaked between 10 to 30 min, and had diminished by 60 min (Fig. 3B). Moreover, we observed a significant elevation in MAP kinase activity after treatment with EPO (data not shown). The kinetics of

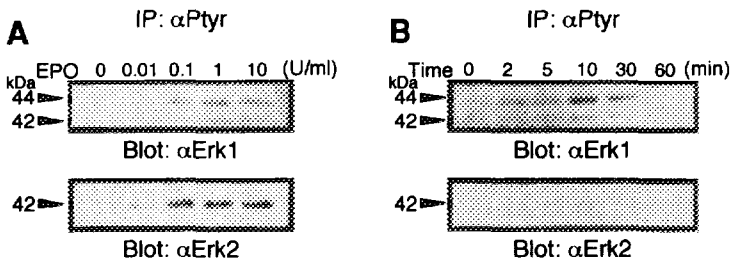

Figure 3. Tyrosine phosphorylation of MAP kinases, Erk1 and Erk2, in response to EPO. UT-7/EPO cells were deprived of growth factors for 24 hrs. After EPO-stimulation, the cells were lysed and phosphotyrosine proteins of the lysates were immunoprecipitated by a monoclonal antibody against phosphotyrosine (4G10), blotted onto nitrocellulose membrane, and probed with anti-Erk1 (upper panel) or anti-Erk2 antibody (lower panel). (A) Starved UT-7/EPO cells were exposed to EPO at increasing concentrations (0-10 U/ml) for 10 min and harvested. The arrows indicate the size of the tyrosine phosphorylated Erk1 and Erk2 proteins. (B) Time course of EPO-induced tyrosine phosphorylation of Erk1 and Erk2 proteins. Starved UT-7/EPO cells were exposed to 10 U/ml of EPO for the times indicated and harvested.

tyrosine phosphorylation and the activation of MAP kinases in EPO-stimulated UT-7/EPO cells were similar to those in TPO-stimulated UT-7/TPO cells.

DISCUSSION

The TPO protein has an EPO domain which has homology with EPO. Genomic analysis of TPO has revealed that the second domain of TPO is also similar to the EPO 3' noncoding sequence (1), suggesting that the two genes have evolved from a common ancestral sequence by duplication. Thus, the proliferation and maturation of megakaryocyte and erythroid precursors is regulated by the structurally and ontogenically related cytokines, TPO and EPO, respectively. It would be interesting to compare the signal transduction of these cytokines to help understand the mechanisms of their specific actions. Many studies showed that EPO rapidly induces tyrosine phosphorylation, and/or activation of phospholipase C-γ1, JAK2, VAV, PI3-K p85, and Shc proteins (3). In accordance with published results from other laboratories (11-13), the activation of MAP kinases by EPO was also observed in a human EPO-dependent erythroid cell line, UT-7/EPO. Very recently Sattler *et al* reported that TPO also induced the tyrosine phosphorylation of p44, which may be Erk1 (7). However, they didn't refer to the tyrosine phosphorylation of Erk2 and activation of the MAP kinases. In this study, we showed that TPO induced the tyrosine phosphorylation of not only Erk1 but also Erk2 protein, and demonstrated that MAP kinase activation was actually induced by TPO.

MAP kinases serve as a signal transducer between the cytoplasm and the nucleus. Activated MAP kinases can enter the nucleus (18) and phosphorylate transcription factors. It is reported that MAP kinase activation is critical in regulating the distinctive effects of epidermal growth factor and nerve growth factor, and that the sustained phosphorylation and activation of MAP kinases may be required for the differentiation of the neurosecretory cell line, PC12 (19). On the other hand, rapid activation of MAP kinases by G-CSF is involved in cellular proliferation (20). Thus, MAP kinases appear to be involved in both proliferation and differentiation. Although Ras-MAP kinase cascades are also activated by several cytokines including IL-3, GM-CSF, EPO and TPO (10-13; present data), their biological significance in hematopoiesis is poorly understood.

Recently, Darnell *et al* have reported that maximal activation of transcription by STAT1 and STAT3 requires both tyrosine and serine phosphorylation (21), although the serine/threonine kinases responsible for the phosphorylation have not yet been identified. Moreover, it was found that STAT1α, STAT3, STAT4, and STAT5 have the highly conserved PMSP amino acid sequence which is similar to MAP kinase's recognition consensus. Recently David *et al* demonstrated that Erk2 activity is required for the induction of early response genes by interferon (IFN)-α and IFN-β through the activation of the STAT1α protein (22). These results raised the possibility that there is crosstalk between the Ras-MAP kinase and JAK-STAT signalling cascades. STAT5 is reportedly activated by EPO and TPO as well as IL-3 and GM-CSF (5, 23-25), and these cytokines can also activate MAP kinases (10-13). Taken together, MAP kinases may also play an important role in the phosphorylation and activation of STAT proteins which are downstream in the cytokine signal transduction pathways.

Although speculative at present, specificity of the molecule(s) downstream of these signalling pathways might account for the distinctive actions of each cytokine. Therefore, it would be of importance to identify the substrates of MAP kinases activated by EPO or TPO. As discussed above, STAT protein(s) may be a candidate for substrates involved in the specific action of these cytokines as it is for IFNs.

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