Thrombopoietin induces activation of at least two distinct signaling pathways

Yuka Nagata, Kazuo Todokoro*

Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), 3-1 Koyadai, Tsukuba, Ibaraki 305, Japan

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Abstract Thrombopoietin (Tpo) is a cytokine regulating megakaryocyte maturation and platelet formation. We studied Tpo-induced signal transduction, and found that Tpo induces phosphorylation of adapter molecules Shc and Vav, and of serine/threonine kinases Raf-1 and mitogen-activated protein (MAP) kinases. Further, Tpo induced activation of Ras, MAP kinase kinase, MAP kinase and Pim-1. Taken together with other observations, we concluded that Tpo induces the activation of at least two distinct signaling pathways, a specific Tyk2-JAK2/STAT1-STAT3-STAT5 signaling cascade and a common Shc/Vav/Ras/Raf-1/MAP kinase kinase/MAP kinase signaling cascade.

Key words: Thrombopoietin; Shc; Vav; Ras; Raf-1; MAP kinase

1. Introduction

Thrombopoietin (Tpo) was recently identified as a specific cytokine, that regulates megakaryocytopoiesis and platelet formation [1–5]. The JAK kinases and STAT proteins play a critical role in Tpo signal transduction [6–8, Nagata et al., unpublished results]. In addition to JAK-STAT signaling pathways, however, cytokines induce activation of a variety of signaling molecules: serine/threonine kinases Raf-1, Pim-1, mitogen-activated protein (MAP) kinases and MAP kinase kinase, SH2 adapter molecules Shc, Grb2 and Vav, GTP-binding protein Ras, protein kinase C-regulating enzymes, phosphatidylinositol 3-kinase and phospholipase Cγ, and tyrosine kinases other than JAK kinase family Fes/Fps, Lck, Lyn and Fyn. These observations suggested that the binding of a cytokine to the receptor induces activation of multiple signaling cascades which cross-talk with each other. Little has been reported about activation of these classical signaling factors in the Tpo-stimulated signaling cascade. We examined which of these signaling factors are actually activated upon Tpo stimulation in Tpo-dependent FD-TPO cells [9], and found that the binding of Tpo to the receptor induces phosphorylation of a Shc and Vav, and activation of Ras, Raf-1, MAP kinase kinase and MAP kinase, but we failed to observe activation of tyrosine kinases other than JAK family. We discuss the role of this common signaling cascade.

2. Materials and methods

2.1. Tpo, antibodies and cell culture

Mouse Tpo cDNA in pME18 was transfected into African green monkey kidney COS-7 cells, and stable transfectants resistant to antibiotic G418 (1 mg/ml) were obtained. COS-7 transfectants expressing Tpo were maintained in DME medium supplemented with 10% fetal calf serum. Conditioned media containing mouse Tpo were prepared by culturing the cells for 2.5 days without fetal calf serum. A mock-transfected COS-7 cell supernatant was also prepared. Tpo-dependent mouse FD-TPO cells [9] were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 units/ml of Tpo. Polyclonal rabbit antibody against mouse Shc and mouse Pim-1 kinase, and mouse monoclonal antibody against phosphotyrosine (4G10) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Polyclonal rabbit antibody against mouse Raf-1 (C-12), and mouse Vav (C-14) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-Ras antibody Y13-259 was from Oncogene Science, Inc. (Uniondale, NY). Mouse monoclonal antibody against MAP kinase (Z033) was from Zymed Laboratories (South San Francisco, CA).

2.2. Immunoprecipitation and immunoblotting

FD-TPO cells were starved in medium containing 0.4% fetal calf serum, 0.125 mg/ml of transferrin and 0.01% BSA without Tpo for 12 h, and restimulated with or without 50 units/ml of Tpo for 20 min in the presence of 0.7 mM sodium orthovanadate. The stimulated and unstimulated cells were immediately lysed in a lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5, 2 mM Pefabloc, 10 ng/ml leupeptin and 10 ng/ml aprotinin). Insoluble material was then removed by centrifugation and the clarified cell lysate was incubated with a specific antibody at 4°C for 2 h. The immunocomplexes were then bound to protein A-Sepharose at 4°C for 1 h. The beads were washed 5 times with lysis buffer containing 0.1% Nonidet P-40 before being boiled in Laemmli sample buffer. Samples were fractionated in 7–12% SDS-polyacrylamide gels and electrotransfered to ECL membrane (Amersham). The membrane was blocked in 5% BSA in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.5% Tween 20 (TBS-T), and incubated with anti-phosphotyrosine antibody (4G10) for 2 h. After washing 3 times with TBS-T, the membrane was incubated with anti-mouse IgG conjugated horseradish peroxidase antibody, and the antibody complexes were visualized by ECL (Amersham). To reprobe the membranes with another antibody, the membranes were washed in the stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH6.7) at 50°C for 30 min, and blocked with BSA.

2.3. Labeling of cells with [^{32}P]orthophosphate

FD-TPO cells (1×10^7) were starved in RPMI 1640 medium with 0.4% BSA for 10 h, and washed with phosphate-free RPMI medium containing 0.4% BSA and 0.5 mM sodium orthovanadate. The cells were incubated for an additional 2 h in 1 ml of this medium containing 1.0 mCi of carrier-free [^{32}P]orthophosphate (Amersham). Labeled cells were cultured with or without Tpo for an additional 7 min and lysed. Cell lysates prepared were normalized for protein content. Immunoprecipitation was performed as described above and the precipitated proteins were resolved by 7.5% SDS-PAGE and autoradiography.

2.4. Assay of Ras activation

Ras activity was assayed by measuring guanine nucleotides bound to Ras by the modified method of Downward et al. [10]. FD-TPO cells were labeled with [^{32}P]orthophosphate, and incubated with or without 50 units/ml of Tpo for 7 min. Cells were lysed by lysis buffer (20 mM Tris-HCl, pH 7.5, 20 mM magnesium chloride, 150 mM sodium chloride, 0.5% Triton X-100, 20 μg/ml aprotinin, and 1 mM sodium orthovanadate). Ras was immunoprecipitated with the anti-Ras-specific

*Corresponding author. Fax: (81) (298) 36 9090.
E-mail: todokoro@rtcs1.riken.go.jp

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monoclonal antibody Y13-259 precoupled to protein A agarose by rabbit anti-rat IgG, and washed. The bound nucleotides were eluted by incubating at 65°C for 5 min in 20 mM Tris-HCl, pH 7.5, 1 mM GTP, 1 mM GDP, 20 mM EDTA and 2% SDS, and separated by thin layer chromatography on PEI-cellulose plates in 0.75 M potassium phosphate buffer (pH 3.4). Nucleotides were visualized by autoradiography, and GDP and GTP standards were visualized by UV light at 286 nm.

2.5. MAP kinase assay

Cell lysates (30 μg/lane) were applied onto 12% micro-SDS-polyacrylamide gels containing 0.5 mg/ml bovine brain myelin basic protein (MBP) as a substrate for MAP kinases. After electrophoresis, the gels were soaked sequentially: in 20% isopropanol in 50 mM Tris-HCl, pH 8.0 for 1 h; in 5 mM β-mercaptoethanol in 50 mM Tris-HCl, pH 8.0 (Solution A) for 1 h; in 6 M guanidine/HCl in Solution A for 1 h; in 0.04% Tween 40 in Solution A for 16 h; and in 40 mM Hepes-NaOH, pH 7.5, 2 mM dithiothreitol, 0.1 mM EGTA, 15 mM $MgCl_2$ for 30 min. MAP kinase assays were then performed by adding 25 μM ATP (25 μCi [γ-^{32}P]ATP/10 ml) at room temperature for 1 h. The gels were washed in 5% trichloroacetic acid solution containing 1% pyrophosphate until the background radioactivity reached almost zero, then dried, and autoradiographed.

2.6. MAP kinase kinase assay

MAP kinase kinase activity was measured by the method of Matsuda et al. [11]. Briefly, cell extracts were applied onto DEAE-cellulose, and the flow-through fractions were assayed for MAP kinase kinase activity by incubating bacterially expressed inactive *Xenopus* MAP kinase and 50 μM [γ-^{32}P]ATP in 20 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$ and 2 mM EGTA at 20°C for 30 min. The phosphorylated substrates were applied onto 15% SDS-polyacrylamide gel and visualized by autoradiography.

3. Results

3.1. Tpo induces phosphorylation of SH2 adapter molecule Shc

Shc is an SH2-containing adapter protein which is tyrosine phosphorylated by various growth stimulations and is believed to mediate a signal from the receptor to Ras activation [12]. To test the possibility that Shc is involved in Tpo-mediated signal transduction, we examined tyrosine-phosphorylation of Shc after Tpo stimulation. Anti-Shc antibody immunoprecipitated 50 and 56 kDa Shc proteins from FD-TPO cells (Fig. 1A, right panel), and immunoblotting with anti-phosphotyrosine antibody revealed that these Shc proteins were clearly tyrosine-phosphorylated in response to Tpo (Fig. 1A, left panel). The major band between 50 kDa and 56 kDa is due to the heavy chain of the first antibody used for immunoprecipitation.

3.2. Vav is tyrosine-phosphorylated by Tpo

The hematopoietic-specific c-*vav* proto-oncogene product, Vav, possesses an SH2 domain and two SH3 domains, which implies that Vav is involved in tyrosine kinase-mediated signaling pathway [13]. To examine the possible involvement of Vav during Tpo receptor-mediated signal transduction, FD-TPO cells were stimulated with or without Tpo, and the immunoprecipitates of anti-Vav antibody were probed with anti-

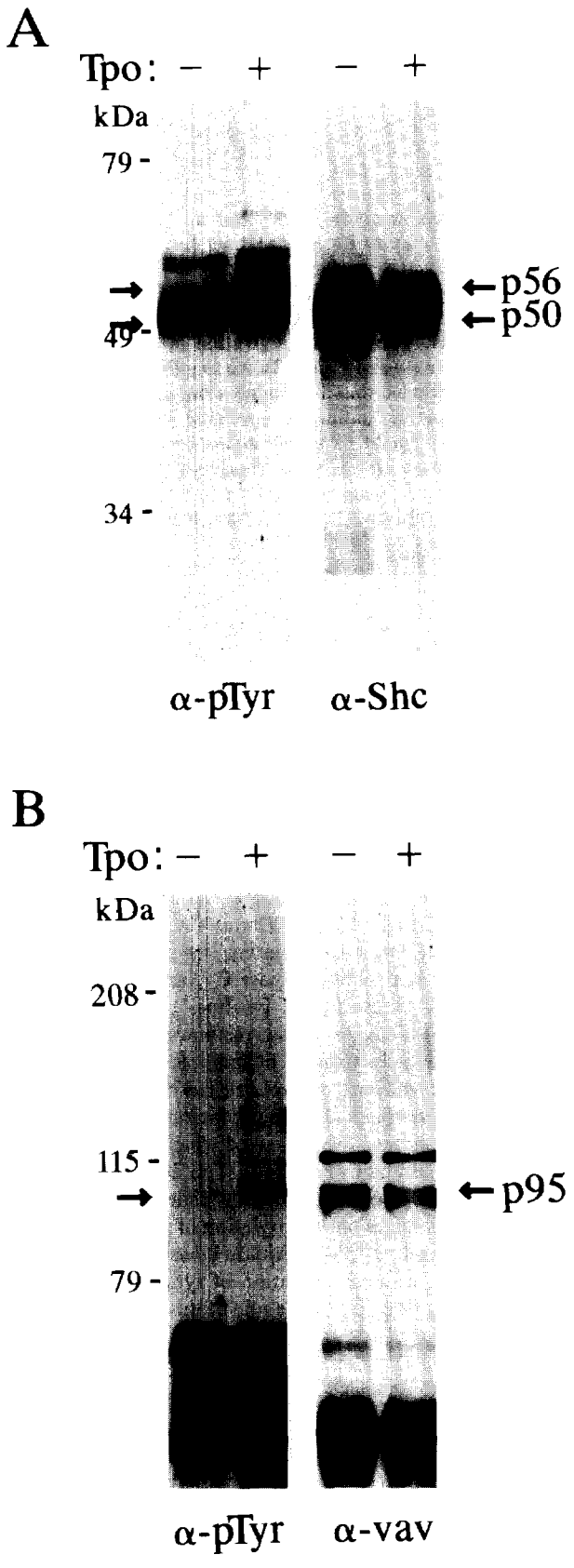

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Fig. 1. Tyrosine-phosphorylation of SH2 adapter molecules Shc and Vav upon Tpo stimulation. (A) Tyrosine-phosphorylation of Shc. Immunoprecipitated Shc form FD-TPO cells stimulated with (+) or without (−) Tpo was immunoblotted with anti-phosphotyrosine antibody (left two lanes) and with anti-Shc antibody (right two lanes). p56 and p50 indicate the immunoprecipitated Shc. The major band between 50 kDa and 56 kDa is the heavy chain of the first antibody used for immunoprecipitation. (B) Tyrosine-phosphorylation of Vav. Vav was immunoprecipitated from FD-TPO cells stimulated with (+) or without (−) Tpo, and probed with anti-phosphotyrosine antibody (left two lanes) and with anti-Vav antibody (right two lanes). p95 indicates phosphorylated Vav.

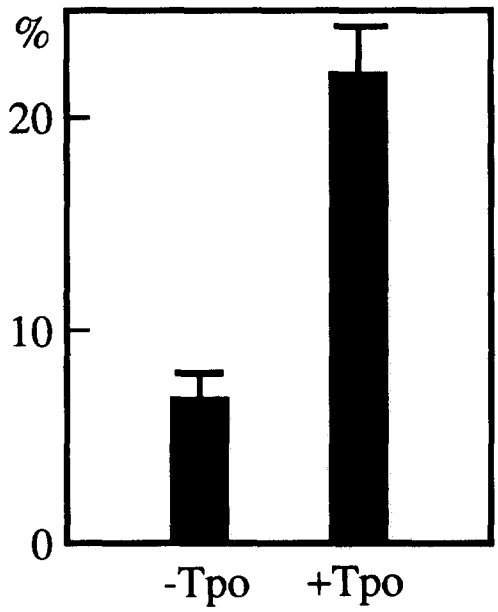

Fig. 2. Activation of Ras by Tpo stimulation. Ras was immunoprecipitated from ^{32}P-labeled FD-TPO cell lysates with anti-Ras antibody. The nucleotides bound to Ras were eluted and separated by thin-layer chromatography. Radioactivities of GDP and GTP bound to Ras were quantified, and the ratios (%) of Ras-GTP/Ras-GDP + Ras-GTP are shown. Values are averages of three independent experiments.

phosphotyrosine antibody. Fig. 1B, left panel, clearly shows that Tpo induced tyrosine phosphorylation of Vav (p95). By reprobing the same blots with anti-Vav antibody (Fig. 1B, right panel), it was confirmed that the degree of tyrosine phosphorylation depends upon Tpo stimulation but not on the amounts of the immunoprecipitated Vav. These results indicate that Vav is phosphorylated on its tyrosine residues in response to Tpo stimulation.

3.3. Activation of Ras by Tpo stimulation

The GTP binding protein, Ras, is involved in a variety of signal transduction pathways [14]. Its activity is regulated by bound guanine nucleotide. The GDP-bound Ras is inactive, whereas the GTP-bound Ras is active. External signals increase the GTP-bound Ras by either stimulating the exchange of GDP with GTP or inhibiting the Ras GTPase activating protein (GAP). To examine if FD-TPO cells could activate Ras in response to Tpo, FD-TPO cells labeled with [^{32}P]orthophosphate were stimulated with or without Tpo, and the ratio (%) of Ras-GTP/Ras-GDP + Ras-GTP were measured. Tpo stimulated the formation of active Ras-GTP form over inactive Ras-GDP form (Fig. 2), and the Ras-GTP form increased in 1 min and peaked in 5–10 min after stimulation (data not shown), indicating that Ras can be activated by Tpo stimulation.

3.4. Raf-1 is phosphorylated by Tpo

A serine/threonine kinase, Raf-1, which specifically associates with activated Ras and MAP kinase kinase [15,16], can be phosphorylated on its tyrosine and serine residues. To examine the possibility that Raf-1 is phosphorylated by Tpo stimulation, cell lysates stimulated with or without Tpo were examined for a change in the mobility of Raf-1 by immunoblotting analysis using an anti-Raf-1-specific antibody (Fig. 3A). In untreated cells, the mobility of Raf-1 was characteristic of its unphosphorylated state (Fig. 3A, left lane). After Tpo stimulation, Raf-1 was largely retarded (shown as p74) (Fig. 3A, right lane), suggesting that Raf-1 is phosphorylated. Furthermore, cells were labeled with [^{32}P]orthophosphate in vivo, stimulated with or without Tpo, and Raf-1 was immunoprecipitated by anti-Raf-1 antibody. Fig. 3B shows that Raf-1 (p74) was weakly but definitely phosphorylated upon Tpo stimulation, indicating that the shift in Raf-1 mobility is due to phosphorylation.

3.5. Activation of MAP kinase kinase and MAP kinase by Tpo stimulation

The activity of MAP kinase kinase, which regulates MAP kinases and is the convergence point of various signals [17] was measured. As shown in Fig. 4A, MAP kinase kinase prepared from Tpo-stimulated cells clearly phosphorylated *Xenopus* MAP kinase, while one from unstimulated cells did not, indicating that a Tpo-mediated signal significantly stimulated MAP kinase kinase activity.

MAP kinase can be activated by tyrosine and threonine phosphorylation through MAP kinase kinase and Raf-1 kinase [17]. Cell extracts stimulated with or without Tpo were probed with anti-MAP kinase antibody, which reacts with both ERK1 and ERK2. As shown in Fig. 4B, right panel, both phosphorylated ERK1 (pERK1) and phosphorylated ERK2 (pERK2) were clearly detected above the unphosphorylated forms in cells stimulated with Tpo, but not in cells without Tpo. Reprobing the same blots with anti-phosphotyrosine antibody confirmed that both pERK1 and pERK2 were tyrosine-phosphorylated (Fig. 4B, left panel).

We next examined whether MAP kinase is actually activated by Tpo, by using the kinase detection assay within gels containing MBP. It was found that MAP kinase activity was strikingly high in Tpo-stimulated cells (Fig. 4C), while little enzyme activity was detected in cells without Tpo. MAP kinase activity increased in 2 min and peaked in 5–10 min after Tpo stimulation (data not shown). Thus, it was concluded that Tpo markedly stimulates the phosphorylation of both ERK1 and ERK2, and that MAP kinase cascade is activated by Tpo.

3.6. Induction of Pim-1 kinase expression by Tpo stimulation

The *pim-1* proto-oncogene encodes a 38 kDa cytoplasmic

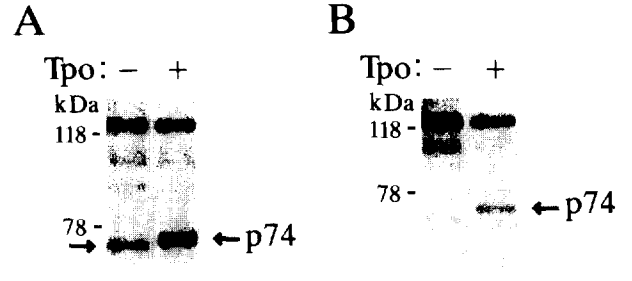

Fig. 3. Phosphorylation of Raf-1 by Tpo stimulation. (A) Mobility shift of Raf-1 after Tpo stimulation. Raf-1 was immunoprecipitated from the FD-TPO cell lysates stimulated with (+) or without (–) Tpo, applied onto SDS-PAGE, and immunoblotted with the same antibody. p74 indicates mobility shifted Raf-1. (B) Phosphorylation of Raf-1. Cells were labeled with [^{32}P]orthophosphate, stimulated with (+) or without (–) Tpo, and Raf-1 (p74) was immunoprecipitated with anti-Raf-1 antibody. Samples were analyzed by SDS-PAGE and autoradiographed.

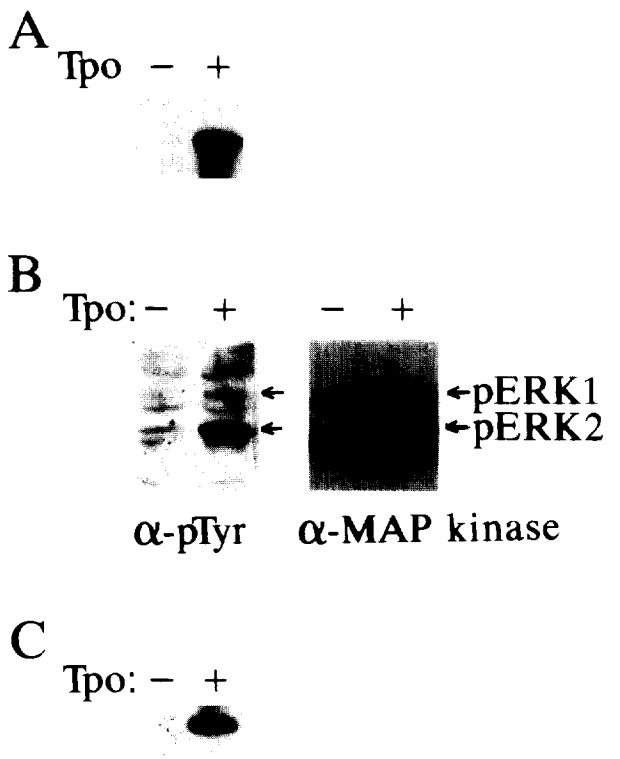

Fig. 4. Activation of MAP kinase kinase and MAP kinase by Tpo receptor-mediated signal. (A) Activation of MAP kinase kinase by Tpo. FD-TPO cells were stimulated with (+) or without (−) Tpo, and cell extracts were assayed for MAP kinase kinase activity after chromatography on DEAE-cellulose. The activity was measured by the ability to phosphorylate recombinant inactive MAP kinase. (B) Tyrosine-phosphorylation of MAP kinase. Cells were stimulated with (+) or without (−) Tpo, and MAP kinases were immunoblotted with anti-MAP kinase (ERK1 and ERK2)-specific antibody (right panel), and reprobed with anti-phosphotyrosine antibody (left panel). (C) MAP kinase is activated by Tpo. MAP kinase assay of the cells stimulated with (+) or without (−) Tpo. MAP kinase activity was measured by the kinase detection assay within gels containing bovine MBP.

serine/threonine kinase which is expressed in hematopoietic cells [18]. As shown in Fig. 5, immunoblot analysis of total cell lysates with anti-Pim-1 kinase antibody revealed that the amounts of Pim-1 kinase (p38) significantly increased in 4 h after Tpo stimulation in FD-TPO cells. Thus, it was concluded that Tpo receptor-mediated signal induces expression of Pim-1 kinase.

4. Discussion

It has been described that Tpo induces the activation of two JAK kinases, JAK2 and Tyk2, and three STAT proteins, STAT1, STAT3 and STAT5 [24–26, Nagata et al., unpublished results]. Furthermore, we reported that the Tpo receptor-mediated lineage-specific signals activate the expression of GATA-1 and NF-E2 genes, and consequently induce expression of the megakaryocyte/platelet-specific cell surface antigens including GPIIIa and the Pm-1 recognized antigen [9]. We therefore concluded that the binding of Tpo to its receptor results in the activation of at least two distinct signaling pathways, a common Shc-Vav-Ras-Raf-1-MAP kinase kinase-MAP kinase signaling cascade and a specific JAK-STAT signaling cascade, as well as a lineage-specific signaling cascade.

Although Tpo receptor lacks tyrosine kinase domains, the binding of Tpo to its receptor induced tyrosine and serine/threonine phosphorylation of various cellular target proteins. Tec-related kinases (Tec, Btk and Itk) are expressed in various hematopoietic cells [19], and are phosphorylated by IL3, stem cell factor and IL6. Syk kinase is also abundantly expressed in mature platelets, and plays an important role in platelet-specific functions [20]. The JAK kinase family might not be the only tyrosine kinase regulating Tpo receptor-mediated signals. Therefore, we examined the possible involvement of Tec kinase and Syk kinase in Tpo receptor-mediated signaling pathways. In our hands, however, we failed to detect tyrosine-phosphorylation of either Tec kinase or Syk kinase (data not shown), although they are expressed in FD-TPO cells. We cannot exclude the possibility that Tec and Syk kinases are involved in Tpo signaling cascades, but they are, at least, not major tyrosine-phosphorylated proteins in FD-TPO cells.

The protein kinase C cascade might be also activated through phosphatidylinositol-3 kinase and phospholipase Cγ, which may finally result in c-*myc* activation. While we detected the expression of both phosphatidylinositol-3 kinase and phospholipase Cγ in FD-TPO cells, we failed to detect phosphorylation of these enzymes even after Tpo-stimulation. This may be due to the weak phosphorylation and/or the antibodies used here, but our result is consistent with a report of Drachman et al. [6]

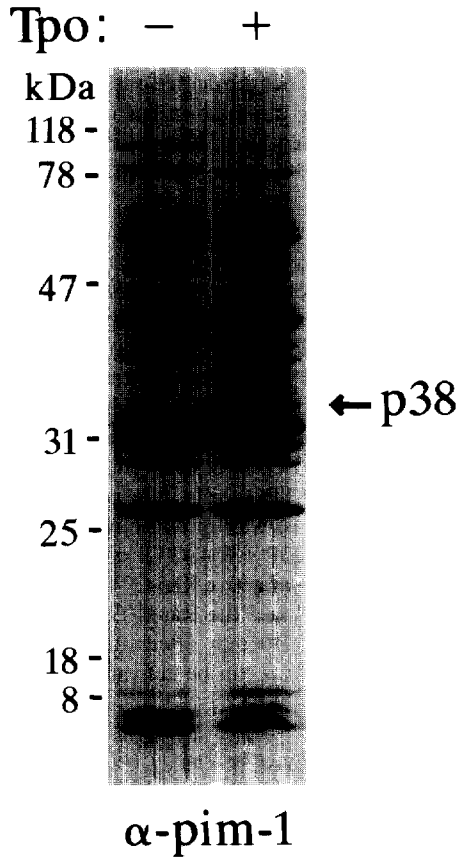

Fig. 5. Induction of Pim-1. FD-TPO cells were stimulated with (+) or without (−) Tpo for 4 h, and total cell lysates were immunoblotted with anti-Pim-1 antibody.

that also failed to see the phosphorylation of phosphatidylinositol 3-kinase and phospholipase Cγ in BaF3 cells expressing exogenous Mpl.

Vav is known to contain an SH2 domain and two SH3 domains [13]. Very recently, it was found that the SH2 domain in Vav is constitutively associated with JAK kinases [21], and that the amino terminal SH3 in Vav constitutively binds to Grb2 [22,23]. It is still controversial, however, whether Vav functions as a guanine exchange factor for Ras or Ras-related proteins Rac and Rho. This makes it essential to clarify the role of Vav in mitogenesis and cytokine receptor signaling in hematopoietic cells, although it is evident that Vav acts on the signaling pathway from the receptor to Ras activation.

There must be Tpo-specific signaling cascades for determining the cell fate of megakaryocytes, and for triggering the polyploidization and platelet release. Nothing is known about what determines megakaryocyte specificity, and what is the difference between erythroid and megakaryocyte in differentiation signals and in transcription factors. These problems await resolution.

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