Thrombopoietin Induces Tyrosine Phosphorylation of Stat3 and Stat5 in Human Blood Platelets

By Yoshitaka Miyakawa, Atsushi Oda, Brian J. Druker, Hiroshi Miyazaki, Makoto Handa, Hideya Ohashi, and Yasuo Ikeda

Thrombopoietin is known to be essential for megakaryocytogenesis and thrombopoiesis. Recently, we and others have shown that thrombopoietin induces rapid tyrosine phosphorylation of Jak2 and other proteins in human platelets and BaF3 cells, genetically engineered to express c-Mpl, a receptor for thrombopoietin. The Jak family of tyrosine kinases are known to mediate some of the effects of cytokines or hematopoietic growth factors by recruitment and tyrosine phosphorylation of a variety of Stat (signal transducers and activators of transcription) proteins. Hence, we have investigated whether Stat proteins are present in platelets and, if so, whether they become tyrosine phosphorylated in response to thrombopoietin. We immunologically identified Stat1, Stat2, Stat3, and Stat5 in human platelet lysates. Thrombopoietin induced tyrosine phosphorylation of Stat3 and Stat5 in these cells. Thrombopoietin also induced tyrosine phosphorylation of Stat3 and Stat5 in FDCP-2 cells genetically engineered to constitutively express human c-Mpl. Thus, our data indicate that Stat3 and Stat5 may be involved in signal transduction after ligand binding to c-Mpl and that this event may have a role in megakaryocytogenesis/thrombopoiesis or possibly a mature platelet function such as aggregation.

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Materials and Methods

Materials. Prostaglandin E1 (PGE1), epinephrine, aspirin, apyrase (type VIII), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), sodium dodecyl sulfate (SDS), 2-mercaptoethanol, sodium orthovanadate, chicken egg albumin, protein A-Sepharose, antimouse IgG-conjugated agarose, Triton X-100, and Tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma (St Louis, MO). Polyvinylidene difluoride (PVDF) membrane (pore size, 0.45 mm) was from Millipore Corp (Bedford, MA). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) molecular standards were from Amersham (Arlington Heights, IL).

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chemiluminescence (ECL) reagents, including second antibodies, were purchased from Amersham. Antiphosphotyrosine murine monoclonal antibody (4G10) was used as described. Anti-Jak2 rabbit polyclonal antibodies were from UBI (Lake Placid, NY). Specific anti-Stat1, -Stat2, -Stat5, and -Stat6 antibodies and Rous sarcoma virus-transformed (RSV)-NIH-3T3 cell lysates, a positive con-

![Image of a diagram showing the comparison of protein bands with different treatments and antibody detection methods.](image)

**Fig 1.** (A) Tyrosine phosphorylation of Stat3 in platelets stimulated by thrombopoietin (100 ng/mL). Platelets were lysed by the addition of an equal amount of a buffer containing 2% Triton X-100 before and after exposure to thrombopoietin (100 ng/mL). Stat3 was immunoprecipitated with specific anti-Stat3 antisera. Immune complexes were lysed with SDS-sample buffer. Immunoblots were probed with antiphosphotyrosine antibody and bands were visualized by chemiluminescence. Lane 1, resting platelets. Lanes 2 through 6, 15 seconds, 1 minute, 5 minutes, 10 minutes, and 30 minutes after exposure to thrombopoietin (100 ng/mL). (B) The same PVDF membrane used in (A) was stripped of the antibody and reprobed for Stat3. Bands were visualized by chemiluminescence. Lanes are the same as in (A). (C) Anti-Stat3 recognized the same protein in lysates from platelets and RSV-3T3 cells. Platelets were lysed by the addition of an equal amount of 2× SDS-sample buffer. After boiling for 5 minutes, proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Stat3 was detected by a specific antibody. Lane 1, resting platelets (1.5 x 10^7 cells/lane). Lane 2, RSV-3T3 cells (20 μg protein/lane).

**Fig 2.** (A) Protein tyrosine phosphorylation of Stat5 in platelets stimulated by thrombopoietin (TPO 100 ng/mL). Platelets were lysed in SDS and lysates were diluted 10-fold. Tyrosine phosphorylated proteins were immunoprecipitated with 4G10. After boiling for 5 minutes in SDS-sample buffer, proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Stat5 was detected by a specific antibody. Lane 1, resting platelets. Lanes 2 through 5, 15 seconds, 1 minute, 5 minutes, and 10 minutes after exposure to thrombopoietin (100 ng/mL). HC, heavy chain of IgG. (B) Protein tyrosine phosphorylation of Jak2 in platelets stimulated by thrombopoietin (TPO 100 ng/mL). Platelets were lysed as in Fig 1. Tyrosine phosphorylated proteins were immunoprecipitated with 4G10. After boiling for 5 minutes, proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Jak2 was detected by a specific antibody. Lanes are the same as in (A). (C) Anti-Stat5 recognized the same protein in lysates from platelets and RSV-3T3 cells. The same as Fig 1C except that Stat5 was detected by a specific antibody. Lane 1, resting platelets (1.5 x 10^7 cells/lane). Lane 2, RSV-3T3 cells (20 μg protein/lane).

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Jak2 in FDCP-hMPL5 cells stimulated by thrombopoietin (100 ng/mL). Stat3 was immunoprecipitated with specific anti-Stat3 antisera. Immune complexes were lysed with SDS-sample buffer. Tyrosine phosphorylation of Stat3 was detected as described in Fig 1A. Lanes 2 through 6, 1 minute, 5 minutes, 10 minutes, 30 minutes, and 60 minutes after exposure to thrombopoietin (100 ng/mL). (B) The same PVDF membrane used in (A) was stripped of the antibody and reprobed with anti-Stat3 antisera. Bands were visualized by chemiluminescence. Lanes are the same as in (A). (C) Tyrosine phosphorylation of Jak2 in FDCP-hMPL5 cells stimulated by thrombopoietin (100 ng/mL). Jak2 was immunoprecipitated and its tyrosine phosphorylation was detected as described in Fig 1A. Lane 1, resting cells. Lanes 2 through 6, 1 minute, 5 minutes, 10 minutes, 30 minutes, and 60 minutes after exposure to thrombopoietin (100 ng/mL). The same lysates as used in (A) and (B) were used. Lanes are the same as in (A). (D) Tyrosine phosphorylation of Stat5 in FDCP-hMPL5 cells stimulated by thrombopoietin (100 ng/mL). Tyrosine phosphorylated proteins were immunoprecipitated with 4G10. After boiling for 5 minutes, proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Stat5 was detected by a specific antibody. Lanes 1, resting cells. Lanes 2 through 6, 1 minute, 5 minutes, 10 minutes, 30 minutes, and 60 minutes after exposure to thrombopoietin (100 ng/mL). The same lysates as used in (D). Jak2 was detected by a specific antibody. Lanes are the same as in (D).

**Platelet preparation.** Human blood from healthy volunteers was drawn by venipuncture into 1/10 volume of 3.8% (wt/vol) trisodium citrate and gently mixed. Platelet-rich plasma (PRP) was prepared by centrifuging the whole blood at 200g for 20 minutes and aspirating PRP. PRP was incubated with aspirin (2 mmol/L) for 30 minutes at room temperature. PGE1 (1 μmol/L) was added from a stock solution in absolute ethanol (1 mmol/L). The PRP was spun at 800g to form a soft platelet pellet. The pellet was resuspended in 1 mL of a modified HEPES-Tyrode buffer (129 mmol/L NaCl, 8.9 mmol/L NaHCO3, 0.8 mmol/L KH2PO4, 0.8 mmol/L MgCl2, 5.6 mmol/L dextrose, and 10 mmol/L HEPES, pH 7.4) also containing apyrase (2 U/mL) and washed twice. Platelets were resuspended in the same buffer at a concentration of 3 × 10⁸ cells/mL with apyrase (2 U/mL) containing 1 mmol/L CaCl2 at 37°C. To make hirudin PRP, hirudin (final concentration, 100 U/mL) was mixed with whole blood instead of citrate.

**Cell lines.** Murine IL-3–dependent myeloid cell line FDCP-2 (kindly provided by Dr Arinobu Tojo, Institute of Medical Science, University of Tokyo, Tokyo, Japan) was maintained in IMDM containing 10% fetal calf serum (FCS) and 1 ng/mL murine IL-3. A thrombopoietin-dependent cell line (FDCP-hMPL5) was established by transforming FDCP-2 cells with an expression plasmid containing a modified HEPES-Tyrode buffer (129 mmol/L NaCl, 8.9 mmol/L NaHCO3, 0.8 mmol/L KH2PO4, 0.8 mmol/L MgCl2, 5.6 mmol/L dextrose, and 10 mmol/L HEPES, pH 7.4) also containing apyrase (2 U/mL) and washed twice. Platelets were resuspended in the same buffer at a concentration of 3 × 10⁸ cells/mL with apyrase (2 U/mL) containing 1 mmol/L CaCl2 at 37°C. To make hirudin PRP, hirudin (final concentration, 100 U/mL) was mixed with whole blood instead of citrate.

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the full-length human c-mpl cDNA driven by the long terminal repeat of Moloney murine sarcoma virus. This cell line expresses human c-Mpl protein on its cell surface and can grow in the presence of recombinant thrombopoietin in a dose-dependent manner. FDCP-2 cells expressing murine erythropoietin receptors (FD-ER3 cells) were established as described previously. Before treatment of the cells with thrombopoietin or erythropoietin, they were incubated in IMDM containing 10% FCS without exogenous IL-3 for 6 hours. After two washes, they were incubated in phosphate-buffered saline (PBS; pH 7.4).

Gel electrophoresis and Western blotting to detect tyrosine phosphorylated proteins or Stat proteins. Platelet stimulation was terminated by the addition of an equal volume of 2X concentrated Laemmli’s sample buffer (10% glyceral, 1% SDS, 5% 2-mercaptoethanol, 50 mmol/L Tris-HCl [pH 6.8], and 0.002% bromophenol blue), 10 mmol/L EGTA, and 1 mmol/L sodium orthovanadate. After boiling at 95°C for 5 minutes, one-dimensional SDS-electrophoresis was performed on 10% or 12% polyacrylamide gels as described. Separated proteins were electrophoretically transferred from the gel onto PVDF membranes in a buffer containing Tris (25 mmol/L), glycine (192 mmol/L), and 20% methanol at 0.2 amps for 12 hours at room temperature. To block residual protein binding sites, transfer membranes were incubated in TBST (Tris-buffered saline [TBS], 10 mmol/L Tris, 150 mmol/L NaCl, pH 7.6, with 0.1% Tween 20) with 10% chicken egg albumin. The blots were washed with TBST and incubated overnight with primary antibodies at a final concentration of 1.0 μg/mL in TBST. The primary antibody was removed and the blots were washed four times in TBST and incubated with horseradish peroxidase-conjugated secondary antibody diluted 1:3,000 in TBST. Blots were then washed four times in TBST. Antibody reactions were detected with chemiluminescence according to the manufacturer’s instructions.

Immunoprecipitation. Cell stimulation was terminated by the addition of an equal amount of lysis buffer (15 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L PMSF, 10 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 0.8 mg/mL leupeptin, 2% Triton X-100 [vol/vol], pH 7.4). After 20 minutes on ice, the lysates were centrifuged at 10,000g (at 4°C) for 20 minutes. The supernatant was removed and incubated with preimmune serum and protein A-Sepharose (40 μL of 50% slurry) for 1 hour. The desired polyclonal or monoclonal antibodies were then added and incubated for 2 to 3 hours on ice. Protein A-Sepharose or antiserum IgG-conjugatedagarose (40 μL of 50% slurry) was added and incubated for several hours. The immune complex was washed with 1 mL of cold washing buffer (15 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L PMSF, 10 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 0.8 mg/mL leupeptin, 1% Triton X-100 [vol/wt], pH 7.4) three times and then lysed with Laemmlı’s sample buffer. When immunoprecipitation of denatured proteins was necessary, cells were lysed first in 1% SDS and boiled for 5 minutes. The lysates were diluted 10-fold in the lysis buffer that contains 1% Triton X-100 before immunoprecipitation.

Measurement of platelet aggregation. The measurement of platelet aggregation was performed with an aggregometer (Hema Tracer TM Model 601; Niko Bio Science, Tokyo, Japan) with continuous stirring (1,000 rpm).

RESULTS

In preliminary experiments, we found that human platelets express Stat1, Stat2, Stat3, and Stat5 but not Stat4 or Stat6 by immunoblotting with specific antisera. A positive control cell lysate (RSV-3T3) was used to confirm that the Stat proteins were detected with the specific antisera and that the proteins detected in platelets comigrated with the Stat proteins in the control lysates (Figs 1C and 2C and data not shown).

We next examined whether any of the Stat proteins became tyrosine phosphorylated after treatment of platelets with thrombopoietin. Platelets treated with thrombopoietin (100 ng/mL) for various times were lysed in a buffer containing 1% Triton X-100 and immunoprecipitated with specific Stat3 antisera. The same amount of Stat3 was detected in each lane (data not shown). (B) Tyrosine phosphorylation of Stat5 in FD-ER3 stimulated by erythropoietin (15 U/mL). Tyrosine phosphorylated proteins were immunoprecipitated with 4G10. After boiling for 5 minutes, proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Stat5 was detected by a specific antibody. Lane 1, resting cells. Lane 2, 10 minutes after exposure to erythropoietin (15 U/mL).
Jak2 has been reported to phosphorylate Stat5 on tyrosine in vitro and possibly in vivo. Consistent with our previous observations, increased amounts of Jak2 were detected in antiphosphotyrosine immunoprecipitates after stimulation of platelets with thrombopoietin (Fig 2B). Tyrosine phosphorylation of Jak2 occurred over a similar time course to that of Stat3 and Stat5 (compare Fig 1A and Fig 2A and B).

To further confirm the link between thrombopoietin stimulation and Stat3/Stat5, we examined whether Stat3 and Stat5 were tyrosine phosphorylated in FDCP-2 cells expressing human c-Mpl (FDCP-hMPL5). FDCP-hMPL5 cells were treated with thrombopoietin (100 ng/mL) for various times and were lysed in a buffer containing 1% Triton X-100. The same amount of a 95-kD protein recognized by Stat3 antisera was immunoprecipitated from all cellular lysates (Fig 3B). This same protein became tyrosine phosphorylated after treatment with thrombopoietin (Fig 3A). We also found that Jak2 was tyrosine phosphorylated after treatment with thrombopoietin (Fig 3C), which corroborates the data obtained in platelets and BaF3 cells transfected with murine c-Mpl. Furthermore, tyrosine phosphorylation of Jak2 occurred along with that of Stat3 (compare Fig 3A and C). We also found that 4G10 immunoprecipitates contained increased amounts of Stat5 and Jak2 from lysates of FDCP-hMPL5 cells (Fig 3D and E) after thrombopoietin stimulation. As in platelets, we did not detect any tyrosine phosphorylation of Stat1 or Stat2 either before or after stimulation of platelets with thrombopoietin (data not shown).

Because of the structural similarity between thrombopoietin and erythropoietin, we also examined whether Stat3 and Stat5 were tyrosine phosphorylated in FDCP-2 cells expressing erythropoietin receptors (FD-ER3 cells). Erythropoietin induced tyrosine phosphorylation of Stat5 but not of Stat3 in these cells (Fig 4A and B).

We have found that treatment of platelets with thrombopoietin enhances aggregation induced by various agonists such as shear stress, epinephrine, and ADP (manuscript submitted). The effect was observed at 1 ng/mL of thrombopoietin and reached a maximum at 10 ng/mL. Figure 5A shows...
that tyrosine phosphorylation of Stat3 also occurs at 1 ng/mL and reached a maximum at 10 ng/mL. We found that epinephrine, which does not induce platelet aggregation in hirudin-PRP,25 does so in the presence of thrombopoietin (manuscript submitted). Figure 5B shows that thrombopoietin (10 ng/mL) did not induce aggregation or shape change. Subsequent addition of epinephrine induced aggregation within 15 seconds. When the order of the agonists was reversed, there was a significant lag time (the lower line). However, aggregation was still observed within 1 minute after the addition of thrombopoietin, suggesting that the priming of epinephrine-induced platelet aggregation occurred within 1 minute.

Finally, we examined whether other Jak family kinases other than Jak2 may also be tyrosine phosphorylated after treatment of platelets with thrombopoietin. We found that thrombopoietin induced tyrosine phosphorylation of Tyk2 in platelets and FDCP-hMPL5 cells (Figs 6 and 7). Under the same conditions, we did not detect any tyrosine phosphorylation of Jak1 and Jak3 (data not shown).

**DISCUSSION**

This report shows the presence of Stat proteins and thrombopoietin-induced tyrosine phosphorylation of Stat3 and Stat5 in human platelets. Both were also tyrosine phosphorylated in FDCP-hMPL5 expressing human c-Mpl, a receptor for thrombopoietin. The tyrosine phosphorylation of Stat proteins induced by thrombopoietin was apparently limited to Stat3 and Stat5. Our data do not rule out the possibility that other unknown Stat-like proteins are also tyrosine phosphorylated in platelets or in FDCP-hMPL5 or that Stat1 or Stat2 could be phosphorylated below the level of detection in our systems. Interestingly, Gurney et al26 showed that Stat 1 and 3 were activated, as measured by binding to a sis-inducible element (SIE), in CMK megakaryoblastoid cells stimulated with thrombopoietin. Tyrosine phosphorylation of Stat1 was not measured directly by these investigators and further studies will be required to explore the possibility that a variety of Stat heterodimers are formed after thrombopoietin stimulation.

In a study by Goullieux et al,24 it was also reported that phosphorylation of tyrosine at position 697 was essential for DNA binding activity of Stat5 and that Stat5 was tyrosine phosphorylated by Jak2 in vitro. Our data show that tyrosine phosphorylation of Jak2 occurred along with that of Stat3 or Stat5. Because phosphorylation of Jak2 is known to be accompanied by enhanced kinase activity,15,18,26 it is likely that Jak2 may at least partially contribute to tyrosine phos-
phosphorylation of the Stat proteins in our system. We have found that, consistent with the report of Rodriguez-Linares et al. Tyk2 and Jak1 are also present in human blood platelets and that Tyk2 becomes tyrosine phosphorylated after stimulation of platelets and FDCP-hMPL5 cells with thrombopoietin (Figs 6 and 7). Thus, Tyk2 may also contribute to tyrosine phosphorylation of the Stat proteins in our system. Recently, Sattler et al. also reported that thrombopoietin induces tyrosine phosphorylation of Tyk2 in Mo7e cells and BaF3 cells, which were genetically engineered to express c-Mpl, although Tortolani et al. did not detect increased tyrosine phosphorylation of Tyk2 in thrombopoietin-treated Mo7e cells. The reason for the different observations is unknown.

Involvement of both Stat3 and Stat5 in signaling induced by thrombopoietin is interesting in view of the recent reports of activation and tyrosine phosphorylation of Stat5 but not of Stat3 induced by erythropoietin (Fig 4), growth hormone, and GM-CSF. Indeed, Stahl et al. determined that the YXXQ motif in gp130 of IL-6 receptor, which is required for tyrosine phosphorylation and activation of Stat3, is absent in the cytoplasmic domain of erythropoietin or GM-CSF receptor. This motif is present in the c-Mpl cDNA used in our transfection studies. Given that the signal transduction pathways used by these cytokines overlap significantly, the involvement of specific combinations of Jak family members and Stat proteins may contribute to the unique effects of each cytokine.

Previously, we showed that thrombin and thrombopoietin induced distinctively different patterns of protein tyrosine phosphorylation in platelets. Although Jak2 may be tyrosine phosphorylated and activated by a few fold in thrombin-stimulated platelets, we did not find measurable increase in tyrosine phosphorylation of Stat3 or Stat5 proteins after thrombin treatment of platelets (data not shown). Thus, the thrombin receptor and c-Mpl appear to use differing signaling pathways and tyrosine phosphorylation of the Stat proteins seems to be unique to the thrombopoietin receptor activation in platelets.

One of the most interesting findings in this study is the presence of Stat proteins in human platelets because they are devoid of nuclei. One possibility is that they are simply remnants from megakaryocytes. However, this would be consistent with a role for Stat proteins in megakaryopoiesis/thrombopoiesis. Another more intriguing hypothesis is that Stat proteins may have roles in platelet activation. Indeed, we found that thrombopoietin-induced tyrosine phosphorylation of Stat3 was observed at concentrations of thrombopoietin of 1 ng/mL and reached a maximum at 10 ng/mL (Fig 5A). In separate experiments, we found that thrombopoietin primed platelet aggregation induced by various agonists in a similar dose range (manuscript submitted). Furthermore, the priming of epinephrine-induced platelet aggregation and tyrosine phosphorylation of Stat3 and Stat5 occurred within 1 minute after stimulation of platelets with thrombopoietin (compare Figs 1A, 2A, and 5B). Because serum levels of thrombopoietin may reach 10 ng/mL in thrombocytopenic patients, these data suggest that tyrosine phosphorylation of Stat3 and Stat5 and priming of platelet aggregation by thrombopoietin occurred in a similar time framework and in a dose range that can be achieved in pathophysiologic states. Because thrombopoietin may enhance the activation of platelets, it is possible that Stat proteins, perhaps through their SH2 domains, could be an element in this enhancement. Most importantly, these data show that thrombopoietin is similar to other cytokines, such as GM-CSF, IL-3, and G-CSF, that are not only required for the proliferation and maturation of progenitor cells but stimulate the functions of mature hematopoietic cells.

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REFERENCES


33. Wittthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, Ihle JN: JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell 74:227, 1993


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