Rapid Communication

Regulation of Platelet Activation In Vitro by the c-Mpl Ligand, Thrombopoietin

By Jichun Chen, Lidija Herceg-Harjacek, Jerome E. Groopman, and Jadwiga Grabarek

Thrombopoietin (TPO) is a recently identified growth factor that regulates megakaryocytopoiesis. Its receptor, c-Mpl, is expressed in megakaryocyte progenitors, mature megakaryocytes, and human blood platelets. We have observed that TPO treatment of human platelets resulted in tyrosine phosphorylation of several cellular proteins, including the c-Mpl receptor and the 85-kD subunit of phosphatidylinositol 3-kinase (PI-3-K). TPO stimulated this tyrosine phosphorylation in a time-dependent manner, reaching a maximum in 5 minutes. The tyrosine phosphorylation of PI-3-K was dependent on the concentration of TPO and reached a maximum at concentrations between 50 and 100 ng/mL. This phosphorylation was independent of extracellular fibrinogen and ligation of the αIIbβ3 integrin. In contrast, TPO, in the presence of exogenous fibrinogen, induced concentration-dependent platelet aggregation, which was blocked by the soluble c-Mpl receptor. Increasing TPO concentrations modulated the degree of the primary wave of aggregation and the lag phase, but not the slope or maximum of the secondary wave of aggregation. This secondary aggregation was controlled by the addition of apyrase, suggesting an adenosine diphosphate (ADP)-dependent mechanism. Treatment of platelets with TPO resulted in augmented binding of 125I-fibrinogen to intact platelets, with a 50% effect (EC50) occurring between 5 and 10 ng/mL. TPO-induced binding of fibrinogen to platelets was comparable in degree with that observed by stimulation with 10 μmol/L ADP. In an immobilized collagen-platelet adhesion assay, a significant increase in the attachment of TPO-stimulated platelets was observed. This effect was dependent on the concentration of TPO. At 50 ng/mL of TPO, platelet attachment to collagen increased threefold compared with the buffer control. Furthermore, the presence of fibrinogen did not significantly alter TPO augmentation of the platelet-collagen interaction. This interaction was mediated by the Arg-Gly-Asp (RGD) adhesion recognition sequence, as it was completely abolished by 100 μmol/L of the RGDS peptide. A fraction of the TPO-dependent platelet attachment to a collagen-coated surface was insensitive to treatment with prostaglandin E1. Furthermore, antibody to αIIb integrin partially inhibited platelet attachment to collagen, suggesting that the integrin αIIbβ3 participates in this association. These data indicate that TPO might function not only as a cytokine in megakaryocyte growth and differentiation, but may also participate in direct platelet activation and modulate platelet-extracellular matrix interactions.

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Regulation of blood cell production, differentiation, and certain functional responses is mediated in part by specific hematopoietic growth factors. The initial event in growth factor action consists of the factor’s binding to its cognate membrane receptor. This binding triggers the activation of a series of intracellular mediators involved in the growth factor’s signaling pathways. Recently, a novel hematopoietic growth factor, termed thrombopoietin (TPO), was cloned and characterized as a primary regulator of megakaryocytopoiesis. Binding of TPO to its receptor, c-Mpl, mediates pleiotropic effects on megakaryocyte development in vitro and in vivo. Studies in rodents and nonhuman primates showed that recombinant TPO was biologically active and was responsible for a significant increase in circulating platelet numbers. Mice rendered null for the c-Mpl receptor through homologous recombination showed a marked reduction in bone marrow (BM) megakaryocytes and blood platelets. As identified by structural homology, the c-Mpl receptor belongs to a cytokine receptor family that lacks intrinsic tyrosine kinase activity. It has been reported previously that the c-Mpl receptor is predominantly expressed in megakaryocytes, endothelium, and platelets. In the present study we show that TPO participates in direct platelet activation. Stimulation of platelets with this cytokine resulted in the tyrosine phosphorylation of platelet proteins, including the c-Mpl receptor and the 85-kD regulatory subunit of phosphatidylinositol 3-kinase (PI-3-K). TPO stimulation of platelets caused the functional modulation of the integrin αIIbβ3, induction of fibrinogen binding, and subsequent platelet aggregation. Pretreatment of platelets with TPO, under conditions that evoked the intracellular changes, significantly increased platelet attachment to a collagen-coated surface. These findings suggest that TPO may serve not only as a regulator of megakaryocytopoiesis, but also as a direct modulator of platelet functional responses.

Materials and Methods

Platelet preparation. Human platelets were prepared as described previously. Briefly, blood drawn from healthy volunteers who had not taken aspirin during the preceding 10 days was collected into 1/10 volume of 3.8% sodium citrate and then centrifuged at 170 g for 17 minutes at room temperature. Platelet-rich plasma (PRP) was removed and centrifuged in the presence of 1 μmol/L prostaglandin E1 (PGE1; Biomol Research Laboratories Inc, Plymouth Meeting, PA) at 800 g for 15 minutes to form a platelet pellet. This pellet was resuspended in a modified HEPES-Tyrode’s buffer, pH 7.4, with 2 U/ml of apyrase (Sigma, St Louis, MO) added. The platelet suspension was layered onto a Sepharose 2B (Pharmacia Biotech, Piscataway, NJ) column equilibrated with HEPES-Tyrode’s buffer, and platelets were then collected at a concentration of 2 to 3 × 10^11 mL.
Western blotting and immunoprecipitation. TPO (a gift from Dr D. Eaton, Genentech, Inc, South San Francisco, CA) was a purified protein with a specific activity of 3 × 10^6 U/mg as determined by the BaF3 cell assay.4 Purified recombinant erythropoietin (EPO) (a gift from Dr A. Sytkowski, Deaconess Hospital, Boston, MA) had a specific activity of 4.3 × 10^5 U/mg. Recombinant human interleukin-3 (rhIL-3; R & D Systems, Minneapolis, MN) was greater than 97% pure with a specific activity of effective dosage (ED50) = 0.1 to 0.4 ng/mL as measured in the TF-1 cell line proliferation assay. Adenosine-5'-diphosphate (ADP) was of a molecular biology grade (Fisher Scientific, Pittsburgh, PA) and bovine thrombin was obtained from Armour Pharmaceutical Company (Kankakee, IL). Washed platelets, obtained as described above, were treated with TPO, ad agonist, or buffer at 37°C and the time specified in the particular experiment. Platelet stimulation was terminated by the addition of an equal volume of two times concentrated Laemmli sample buffer.17,18 The samples were boiled and analyzed by one-dimensional (1-D) electrophoresis on 7.5% polyacrylamide gels. Some samples were also analyzed by isoelectrofocusing 2-D gel electrophoresis, as described below. Platelet protein concentrations were adjusted to 6 to 7 × 10^9 platelets loaded to each gel well. The proteins were transferred from the gels onto a 0.45-μm nitrocellulose membrane for subsequent immunoblot analysis using a specific monoclonal antiphosphotyrosine antibody (Zymed Laboratories Inc, South San Francisco, CA). In phosphoprotein identification studies, a monoclonal antibody (MoAb) specific for the human c-Mpl receptor (Genzyme Inc, Cambridge, MA) and a polyclonal antibody to the 85-kD subunit of PI 3-K (Upstate Biotechnology Inc, Lake Placid, NY) were used. Primary binding of these antibodies was detected using the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL) according to the manufacturer’s instructions. In immunoprecipitation experiments, platelet-agonist interactions were terminated by platelet lysis in 3 times concentrated buffer with final concentrations of 25 mmol/L HEPES, pH 7.4, 100 mmol/L sodium fluoride, 10 mmol/L pyrophosphate, 2 mmol/L sodium orthovanadate, 2 mmol/L EDTA, 2 mmol/L sodium molybdate, 0.15 U/mL aprotinin, 40 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1% Nonidet P-40 (NP-40) (Sigma). Immunoprecipitations were done by incubating samples with a specific antibody or control serum, as described in the individual experiment, and immune complexes were collected by protein A-Sepharose (Pharmacia Biotech). Immunoprecipitates were washed, dissolved by boiling in sodium dodecyl sulfate (SDS) Laemmli sample buffer, and resolved by polyacrylamide gel electrophoresis (PAGE). The immunoprecipitates were subsequently identified with a second antibody by Western blotting as stated above.

Isolelectrofocusing 2-D gel electrophoresis (IEF). Platelet proteins corresponding to lysates of 7 × 10^9 platelets were separated by isoelectrofocusing and sequential second-dimension gel electrophoresis using a Mini-Protean II system (BioRad, Hercules, CA). Briefly, first dimension tube gels, which contained 9.2 mol/L urea, 4% acrylamide-bis acrylamide mixture (17.5:1, w/w), 0.3% ampholytes (2% Biolyte 3/10 [BioRad] and 1% Pharmalyte 8/10 [Pharmacia Biotech]) and 2% NP-40, were focused for 15 minutes at 500 V and for 2 hours at 1,500 V using 50 mmol/L sodium hydroxide and 7 mmol/L phosphoric acid as cathode and anode buffers, respectively. The pH gradient, obtained by the isoelectrofocusing process in the first-dimension tube gel, was analyzed by measuring pH values in different gel sections. The second-dimension electrophoresis was run on 7.5% polyacrylamide SDS slab gels, and immunoblotting was accomplished as described above.

Aggregation of platelets. Platelet aggregation was monitored according to the Born method19 at 37°C at a continuous rate of stirring at 900 rpm in the lumiaggregometer (Chronolog, Havertown, PA). Platelets (2.5 × 10^10 cells/mL) were preincubated with TPO for 5 minutes at 37°C without stirring, then fibrinogen (100 μg/mL) was added to the platelet suspension, and light transmission was recorded. In our experiments, we used Kab-B fibrinogen (Pharmacia Hepar, Franklin, OH), which was depleted of plasminogen by passage through lysine-Sepharose equilibrated in buffer containing 0.05 mol/L HEPES, pH 7.4, and 0.15 mol/L NaCl. A 20-fold excess of the soluble c-Mpl receptor (a gift from Dr D. Eaton, Genentech, Inc) was added simultaneously with fibrinogen to the platelet suspension. The c-Mpl was a purified extracellular fragment of the receptor bound to IgG.

Measurement of adenosine triphosphate release. We applied the luciferin-luciferase technique to measure release of ATP from platelets. Commercially available Chrono-Lume reagent (Chronolog) was used to measure ATP secretion in TPO-stimulated platelets in a lumiaggregometer. The step change in the luminescence before and after aggregation was measured according to the manufacturer’s recommendations with slight modification. One milliliter of platelet suspension (2.5 × 10^9) was preincubated with 100 ng/mL TPO or buffer control, as described for the aggregation experiments. After incubation, 10 μL of reconstituted Chrono-Lume reagent was added to the platelet suspension and luminescence recorded until a stable baseline was obtained. The fibrinogen (100 μg/mL) was then added and the luminescence and aggregation were simultaneously recorded. The amount of ATP released during TPO-stimulated aggregation was calculated by comparing the step change in luminescence induced by TPO to that measured in the platelet suspension containing 2 nmol/L of the ATP standard.

Fibrinogen binding to platelets. Fibrinogen was purified as described above, and labeled with 125Iodine (NEN, Boston, MA) using iododecane (Pierce, Rockford, IL) as recommended by the manufacturer. 125I-fibrinogen binding to intact platelets was measured by the modified filtration technique used in radioligand binding assays.20 Platelets (1.5 × 10^9) in 0.6 mL of reaction mixture containing a range of concentrations of TPO (5 to 100 ng/mL) were incubated for 5 minutes at 37°C and then the 0.16 μmol/L fibrinogen comprising 0.017 μCi of tracer 125I-fibrinogen (with a specific activity of 0.17 μCi/μg) was added. Samples were incubated without stirring for 30 minutes at room temperature with or without a 20-fold excess of cold fibrinogen. After incubation, the bound ligand was separated from the free radioligand in a Millipore Sampling Manifold (Millipore Corp, Bedford, MA) using Whatman glass microfiber filters (Whatman International Ltd, Maidstone, UK). The filters were initially blocked with 1% albumin in HEPES-Tyrode’s buffer at 4°C. The incubation mixtures were placed directly onto the filters, vacuum filtered, and the samples were then rinsed three times with 3 mL each of ice-cold HEPES-Tyrode’s buffer. The filters were removed and the radioactivity was measured. The specific binding was calculated from the total and nonspecific binding (binding in the presence of an excess of cold fibrinogen).

Platelet interaction with immobilized collagen. Platelet association with a collagen-coated surface was assayed by a protocol adapted from a method originally described for measuring platelet-subendothelial extracellular matrix interactions.21 Collagen type I coated 6-well plates (Collaborative Biomedical Products, Bedford, MA), blocked overnight with 0.25% bovine serum albumin (BSA) (Sigma), were washed twice with phosphate-buffered saline (PBS), pH 7.5, and once with HEPES-Tyrode’s buffer, pH 7.4. Platelets, obtained as described above, were labeled by 60-minute incubation with [35S]sodium chromate (DuPont/NEN, N Biljertica, MA) (0.1 mCi/mL) at room temperature. After the removal of free radiosotope by gel filtration through a Sepharose 2B column, platelet numbers were adjusted with HEPES-Tyrode’s buffer to about 2.5 × 10^9/mL. The platelet suspension was then divided into 3-mL aliquots to which either various concentrations (5 to 100 ng/mL) of TPO or an equal volume of PBS, as a control, were added. In parallel experiments, fibrinogen was added to the platelet suspension at a final concentration of 36 μg/mL, which is threefold lower than the concentration...
used in the aggregation experiments. After a 5-minute incubation at 37°C, 100 μM of RGDS or RGES peptides (Sigma), 100 ng/mL of PGE₂, or 20 μg/mL of α₉b MoAb (Immunotech, Inc, Westbrook, ME) were added to the platelet suspension. This MoAb (SZ22) is described by the manufacturer as being reactive with the glycoprotein IIb independent of the presence of glycoprotein IIa. SZ22 does not inhibit fibrinogen binding to human platelets induced by ADP, arachidonic acid or PAF, nor does it inhibit platelet aggregation and secretion induced by collagen, arachidonic acid, or thrombin. One-milliliter samples were pipetted into each well of the collagen-coated plates, which were then gently agitated for 30 minutes at 37°C. Subsequently, the unattached platelets were collected and the wells were washed twice with 1% BSA in HEPES-Tyrode’s buffer. Attached platelets were then lysed with 19 mmol/L Na₂CO₃/0.1 N NaOH, and the radioactivity in the samples was measured using a Beckman Gamma 5500 counter (Beckman, Palo Alto, CA).

RESULTS

The observation that platelets express the c-Mpl receptor suggested that its ligand, TPO, may be involved in receptor-mediated platelet activation. Immunoblot analysis, using an antiphosphotyrosine antibody of the total lysates of washed human platelets (2 x 10⁵ cells/mL) treated with TPO (50 ng/mL) or buffer control for various time periods at 37°C, identified several TPO-dependent tyrosine phosphorylated proteins (Fig 1A). The addition of TPO to platelets induced the most prominent time-dependent tyrosine phosphorylation of proteins migrating at 85- to 90-kD positions, and less obvious tyrosine phosphorylated bands at 52-kD and 125-kD positions. Phosphorylation of the 85- to 90-kD bands following the addition of TPO was observed within 1 minute and reached a maximum between 5 and 8 minutes. The changes in tyrosine phosphorylation after platelet stimulation for 5 minutes with increasing concentrations of TPO were analyzed by Western blotting (Fig 1B), which demonstrated a concentration dependent response. Phosphorylation of the 85- to 90-kD proteins increased above the basal level at a TPO concentration of 5 ng/mL and reached a maximum at 50 to 10 ng/mL. The hematopoietic growth factors EPO (200 ng/mL) or IL-3 (400 ng/mL) did not induce the tyrosine phosphorylation observed with the TPO treatment (data not shown). In further experiments, at the end of 5 minutes of platelet stimulation with TPO at 37°C without stirring, fibrinogen was added and platelets were stirred in an aggregometer for an additional 5 minutes. As evaluated by Western blotting, the presence of fibrinogen and the extended stimulation of platelets under aggregatory conditions did not result in any significant changes in the pattern of tyrosine phosphorylated platelet proteins (Fig 2A). Additionally, no change in the degree of phosphorylation of the 85- to 90-kD proteins was observed under these conditions. The presence of 500 μmol/L RGDS peptide during the treatment of platelets with TPO resulted in no alteration in this tyrosine phosphorylation. Used as a control, 10 μmol/L ADP alone did not evoke the tyrosine phosphorylation of the 85- to 90-kD proteins. Figure 2B shows the result of reprobing the same membrane with PI 3-K specific antibody to demonstrate an equal protein concentration in the analyzed samples. Based on these findings, we propose that platelet protein phosphorylation induced by TPO was time- and concentration-dependent, and had features distinctly different from those obtained by platelet treatment with other cytokines, EPO or IL-3. Additionally, these results suggested a signaling that occurred independently of exogenous fibrinogen, the ligation of α₉bβ₃, or the presence of ADP.

To analyze the phosphoproteins induced by TPO treatment of platelets, we used an antibody specific for the c-Mpl receptor and an antibody against the 85-kD subunit of PI 3-K. This later molecule is a common intermediate in a variety of growth factor receptor signaling pathways. Platelets were stimulated with TPO (50 ng/mL) in the presence or absence of exogenous fibrinogen. At the end of 5 minutes, activated platelets and corresponding controls were lysed and immunoprecipitated with the c-Mpl antibody. The immunoprecipitates were subject to isoelectrofocusing, followed by 2-D gel electrophoresis, and then Western blotting using the phosphotyrosine antibody (Fig 3). The c-Mpl antibody precipitated, from TPO-stimulated platelets, different tyrosine-phosphorylated proteins, each with an 85-kD molecular mass but with distinct PI values of 5.2, 5.7, and 6.2. The immunoprecipitates also contained an 80-kD phosphoprotein with a...
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Effect of fibrinogen, RGDS, and ADP on TPO-stimulated tyrosine phosphorylation in platelets. Platelets were incubated with 50 ng/mL TPO, 10 μmol/L ADP, or an equal volume of buffer at 37°C for 5 minutes. In samples that were treated with TPO in the presence of ADP, both reagents were added to platelets simultaneously and the preincubation was omitted in the thrombin stimulated sample. (A) Platelets were then stirred for another 5 minutes in the presence (+) or absence (-) of 100 μg/mL fibrinogen, 500 μmol/L RGDS, or control buffer. Lanes 1 and 2, PBS control; lane 3, 1 U/mL thrombin; lanes 4 and 5, 50 ng/mL TPO; lanes 6 and 7, 50 ng/mL TPO and 500 μmol/L RGDS; lanes 8 and 9, 10 μmol/L ADP; lanes 10 and 11, 10 μmol/L ADP and 50 ng/mL TPO. The reactions were terminated after 5 minutes with 4 times concentrated Laemmli sample buffer and immunoblotting analysis was achieved as described in Materials and Methods. (B) To insure that all lanes were loaded equally, the same membranes were stripped and reprobed with a polyclonal antibody recognizing the 85-kD subunit of PI 3-K. Bands were visualized with ECL as described in Materials and Methods.

Platelet activation by TPO, as shown by protein phosphorylation, served as an initial indication that TPO may participate in the modulation of platelet functional responses. Stimulation of washed human platelets (2.5 × 10⁹ cells/mL) with various concentrations of TPO (25 to 100 ng/mL) in the presence of fibrinogen induced a concentration dependent primary aggregation and a subsequent secondary wave of aggregation (Fig 5). At 10 minutes, the extent of irreversible secondary platelet aggregation was approximately the same for all samples regardless of the TPO concentration. However, in these studies the TPO concentration did affect the lag phase of the aggregation. The results shown in Fig 5 are from one experiment that is representative of the results obtained from various donors. Subthreshold concentrations of TPO evoking aggregation differed only slightly among the 10 individuals tested. The TPO plus fibrinogen effect on platelet aggregation was specific because it was completely inhibited by the addition of the soluble c-Mpl receptor. Moreover, treatment of platelets with other growth factors, EPO or IL-3, did not produce the same effect (data not shown). TPO alone, even at concentrations as high as 100 ng/mL, did not cause platelet aggregation.

When 2 U/mL of the ADP-degrading enzyme apyrase was added to platelets stimulated with TPO in the presence of fibrinogen, their primary and also secondary aggregation were abolished (data not shown). At lower concentrations (0.2 or 0.02 U/mL) of apyrase, inhibition of primary aggregation and delayed secondary aggregation were observed. Thus, TPO-associated platelet aggregation involved ADP.
The ATP release from dense granules of TPO-aggregated platelets was measured by the luminescence method. Release of ATP (0.5 μmol/10^11 platelets) from platelets aggregated with 100 ng/mL TPO was observed. This release comprised on average 10% of the total content of ATP in 10^11 platelets. These results likely reflect TPO enhancement of platelet sensitivity to traces of ADP in the platelet preparations that resulted in primary aggregation, and which then triggered secondary aggregation and dense granule secretion.

Finally, we investigated the effect of TPO treatment of platelets on their interaction with immobilized collagen. This system provides a model of platelet interaction with an extracellular matrix. Platelets labeled with ^31P were stimulated with TPO or a buffer control for 5 minutes at 37°C and then added onto collagen-coated plates. Enhancement of platelet deposition on a collagen I coated surface in response to various concentrations of TPO (5 to 100 ng/mL) was observed (Fig 7). After incubation, the platelet suspension was examined to ensure that aggregates were not present. TPO had comparable effects on platelet attachment to collagen in the presence and absence of exogenous fibrinogen (Fig 8). The observed increase in platelet attachment to collagen in the presence of both TPO and fibrinogen was the sum of the enhanced association seen with TPO alone and fibrinogen alone. The study of platelet interaction with collagen in the presence of TPO showed, as examined by phase-contrast microscopy, the attachment of single cells as well as platelet aggregates forming thrombus-like structures. These platelet-collagen interactions were almost completely abolished by 100 μmol/L RGDS peptide containing the RGD adhesive sequence present in collagen I, but not by 100 μmol/L RGES, the inactive analog (Table 1). These interactions were only partially inhibited when PGE, an inhibitor of platelet activation, was included in the assay. Treatment with an antibody to the αIβ chain of the integrin also partially inhibited these interactions; this inhibition was greater in the presence of fibrinogen. These observations suggested the involvement of the integrin αIβ in TPO activation-mediated platelet-collagen association.

These results suggest that platelet functional responses after TPO stimulation reflect an initial interaction of TPO with its receptor and a secondary activation of the integrin αIβ, which then mediates the functional effects observed under these conditions.

**DISCUSSION**

TPO appears to be a major physiologic regulator of megakaryopoiesis and has attracted much interest because of its potential therapeutic application for amelioration of thrombocytopenia. To date, most of the biologic characterization of TPO has focused on its role in megakaryocyte proliferation, maturation, and subsequent release of platelets.
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Fig 4. TPO concentration-dependent tyrosine phosphorylation of the 85-kD subunit of PI 3-K. Platelets stimulated with various concentrations of TPO for 5 minutes at 37°C were lysed and then subjected to immunoprecipitation as described in Materials and Methods by using a polyclonal antibody specific for the 85-kD subunit of PI 3-K (5 pg/mL) (lanes 1 through 5) or normal rabbit serum as a control (lane 6). The immunoprecipitates were then Western blotted with antiphosphotyrosine antibody. The same blot was stripped and subsequently immunoblotted with an antibody specific for the 85-kD subunit of PI 3-K. Lane 1, control; lane 2, 5 ng/mL TPO; lane 3, 10 ng/mL TPO; lane 4, 50 ng/mL TPO; lane 5, 100 ng/mL TPO; lane 6, 100 ng/mL TPO.

In this study, we show that TPO directly activates blood platelets, and that this activation shares several distinctive features with those induced by other cytokines in various cell systems. Similar to most receptor-mediated platelet agonists, TPO induced the phosphorylation of platelet proteins. TPO treatment of resting platelets resulted in a striking tyrosine phosphorylation of protein(s) in the 85- to 90-kD molecular mass region. This phosphorylation was time- and concentration-dependent. Moreover, TPO-induced tyrosine phosphorylation was independent of extracellular fibrinogen presence, the occupancy state of its receptor, or the presence of ADP. In a recent report of TPO-induced tyrosine phosphorylation in human platelets, the phosphoproteins with 85- to 95-kD relative molecular masses were noted but not further characterized.

In this work, we examined by phosphotyrosine immunoblotting the c-Mpl specific immunoprecipitates obtained from TPO-treated platelets. These c-Mpl immunoprecipitates, resolved by 2-D gel electrophoresis and probed with a specific antiphosphotyrosine antibody, showed the presence of three 85-kD phosphoproteins with PI values of 5.2, 5.7, and 6.2, respectively, in addition to an 80-kD phosphoprotein with a PI value of 6.2. These observations suggest a receptor-mediated mechanism of TPO stimulation of platelets, and the importance of c-Mpl tyrosine phosphorylation in this event. Interestingly, it also suggests the presence of different forms of the c-Mpl receptor in human blood platelets, and possibly diverse patterns of phosphorylation upon their stimulation. We are currently pursuing additional stud-
TPO-originated cell signal transduction is cell-type specific. A possibility is that different TPO signal transduction pathways exist with other platelet agonists. Another less likely cell types. We observed this phosphorylation after 5 minutes of stimulation of platelets with TPO. This phosphorylation manner with a maximum effect at 50 to 100 ng/mL, a concentration range that in prior studies appeared optimal to induce proliferative and functional changes in BM megakaryocytes in vitro. This observation suggests the association of c-Mpl receptor stimulation with PI 3-K in platelets. The association of the platelet c-Kit receptor and its ligand, the stem cell factor, with the platelet PI 3-K has been previously observed. Thus, PI 3-K activation may represent a common pathway for hematopoietic growth factors in blood platelets. Interestingly, a published report of TPO-induced tyrosine phosphorylation in BaF3 cells expressing the murine c-Mpl receptor identified the association of PI 3-K with the activated c-Mpl in a tyrosine phosphorylation-independent manner. This difference from our data may indicate that TPO-originated cell signal transduction is cell-type specific. Another possibility is that the kinetics of tyrosine phosphorylation of the PI 3-K 85-kD subunit are different in various cell types. We observed this phosphorylation after 5 minutes of stimulation of platelets with TPO. This phosphorylation represents a rather late response in comparison to the usually rapid 5- to 30-second phosphorylation of platelet proteins observed with other platelet agonists. Another less likely possibility is that different TPO signal transduction pathways exist in humans and mice. Further studies with murine platelets can address this issue. Our observations in anucleate blood platelets also suggest that the tyrosine kinase activity and 3-phosphate-containing inositol phospholipids involved in TPO-mediated platelet ac-

**Table 1. Inhibition of Platelet Attachment to Immobilized Collagen I**

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<td>RGDS</td>
<td>81±3</td>
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<td>RGES</td>
<td>0</td>
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<tr>
<td>PGE$_1$</td>
<td>55±8</td>
<td>70±6</td>
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<tr>
<td>Anti-gpllb</td>
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Platelets were obtained and labeled with [35Cr] as described in Materials and Methods. Gel-filtered platelets were then incubated with 50 ng/mL TPO or PBS for 5 minutes at 37°C. After the incubation, 100 μmol/L RGDS or RGES peptides, 100 ng/mL PGE$_1$, or 20 μg/mL anti-gpllb antibody was added followed by 36 μg/mL fibrinogen (Fg) or control buffer. Samples were then immediately transferred to collagen I-coated 6-well plates and gently agitated for 30 minutes at 37°C. The nonattached platelets were subsequently removed, the wells washed, and the attached platelets lysed as described in Materials and Methods. Radioactivity in the samples was measured and the percent inhibition was calculated inside each experiment. The results shown represent a mean of two (anti-gpllb) or three (RGDS/RGES and PGE$_1$) experiments done in triplicate.
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tivation can be dissociated from cellular functions related to TPO-induced proliferation or differentiation. Our prior work with the stem cell factor/kit ligand similarly supports the possibility that hematopoietic growth factors have unique physiologic functions in anucleate platelets.19

Unlike the case with tyrosine phosphorylation that was regulated through the c-Mpl receptor, aggregation of platelets involving TPO resulted from the alteration of the integrin αIIbβ3 and fibrinogen binding. Furthermore, TPO in the presence of fibrinogen, in an ADP-dependent mechanism, induced aggregation of platelets. These data present direct evidence that TPO can regulate platelet functional responses.

A marked augmentation of platelet attachment to immobilized collagen was observed when platelets were prestimulated with various concentrations of TPO before their interaction with collagen. Although the mechanism for this effect may be rather complex,46-47 the present study strongly suggests that TPO stimulated the platelets integrin αIIbβ3 and that this integrin may play a role in the platelet-collagen association. The involvement of this integrin in platelet-collagen interactions has been identified in previous studies.48-52 Our inhibition studies indicated that only part of the TPO-induced attachment of platelets to collagen was inhibited by PGE1. This supports the concept of a secondary activation-dependent platelet adhesion to collagen, an adhesion that is mediated by the integrin αIIbβ3 and is insensitive to PGE1.52,53 Furthermore, an MoAb directed against the αIIb chain of the platelet integrin also inhibited this interaction. This inhibitory effect was noticeably higher in the presence of bound fibrinogen, supporting published data identifying collagen-induced aggregation. These data present direct evidence that TPO can regulate platelet functional responses.

A potential complication of the therapeutic use of TPO is suggested by our study, because TPO was able to induce platelet aggregation and increased platelet attachment to collagen. Such TPO-mediated effects could potentiate thrombosis in situations where platelets encounter collagen and other adhesive substrates. Preclinical animal models of vascular injury are best suited to evaluate the potential enhancement of platelet thrombus formation under TPO treatment.

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