Biochemical and Biophysical Research Communications **261,** 58–63 (1999)
Article ID bbrc.1999.0979, available online at http://www.idealibrary.com on IDEAL®

Thrombopoietin Potentiates Agonist-Stimulated Activation of p38 Mitogen-Activated Protein Kinase in Human Platelets

Yasuharu Ezumi,* Eisuke Nishida,† Takashi Uchiyama,* and Hiroshi Takayama*,[1]

**Department of Hematology and Oncology, Clinical Sciences for Pathological Organs, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan; and †Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan*

Received May 28, 1999

Thrombopoietin (TPO) plays a crucial role in megakaryocyte differentiation and platelet production. c-Mpl, a receptor for TPO, is also expressed in terminally differentiated platelets. We investigated the effects of TPO on activation of p38 mitogen-activated protein kinase in human platelets. Thrombin, a thrombin receptor agonist peptide, a thromboxane A_2 analogue, collagen, crosslinking the glycoprotein VI, ADP, and epinephrine, but not phorbol 12, 13-dibutyrate activated p38. TPO did not activate p38 by itself, whereas TPO pretreatment potentiated the agonist-induced activation of p38. TPO did not promote phosphorylation of Hsp27 and cytosolic phospholipase A_2 by itself, but enhanced thrombin-induced phosphorylation of them. The specific p38 inhibitor SB203580 strongly inhibited such phosphorylation. Thus, TPO possesses the priming effect on p38 activation in human platelets and could affect platelet functions through the p38 pathway. © 1999 Academic Press

Thrombopoietin (TPO), also called the c-Mpl ligand or the megakaryocyte growth and development factor, plays a crucial role in megakaryocyte differentiation and platelet production (1–4). However, c-Mpl is also expressed in terminally differentiated platelets as well as the megakaryocytic progenitors (5). Others and we have reported that TPO does not induce either platelet aggregation or release reaction by itself but potentiates aggregation and release reaction induced by other agonists (6–12). Furthermore, TPO elicits tyrosine phosphorylation of signaling molecules including the Janus tyrosine kinases, JAK2 and Tyk2, in platelets (6–13). However, it has not been studied in detail how TPO affects platelet functions or the signal transduction stimulated by other agonists.

p38 mitogen-activated protein (MAP) kinase is a member of the MAP kinase family (14, 15), which includes the extracellular signal-regulated kinases (ERKs), ERK1 and ERK2, and the Jun N-terminal kinases (JNKs), JNK1 and JNK2. p38 is the mammalian homologue of HOG1 from yeast and mainly activated by stress stimuli and inflammatory cytokines (16, 17). p38 and ERKs but not JNKs have been identified in platelets (18, 19). We have shown that TPO does not activate ERKs or protein kinase C (PKC) by itself but markedly enhances PKC-mediated activation of ERKs induced by thrombin or phorbol esters (20). Previous reports have shown that physiologic agonists including thrombin and collagen induce activation of p38 in platelets (21, 22) and that p38 phosphorylates and activates cytosolic phospholipase A_2 ($cPLA_2$) leading to arachidonic acid release and is involved in platelet aggregation (22, 23). In this report, we investigated the effects of TPO on p38 activation in human platelets.

MATERIALS AND METHODS

Reagents. Affinity-purified rabbit polyclonal antibody against p38 (C-20) and anti-$cPLA_2$ monoclonal antibody (clone 4-4B-3C) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphotyrosine mouse monoclonal antibody (clone 4G10) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse monoclonal antibody (clone G3.1) specific for the small heat shock protein Hsp27 was obtained from StressGen Biotechnologies Corp. (Victoria, BC, Canada). $F(ab')_2$ of IgG specific for glycoprotein

[1] To whom correspondence should be addressed at Department of Hematology and Oncology, Clinical Sciences for Pathological Organs, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawaracho, Sakyo-ku, Kyoto 606-8507, Japan. Fax: +81-75-751-3201. E-mail: hiro@kuhp.kyoto-u.ac.jp.

Abbreviations: TPO, thrombopoietin; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; PKC, protein kinase C; $cPLA_2$, cytosolic phospholipase A_2; GP, glycoprotein; ATF, activating transcription factor; STA_2, 9, 11-epithio-11, 12-methano-thromboxane A_2; PDBu, phorbol 12, 13-dibutyrate; TRAP, thrombin receptor agonist peptide; PAGE, polyacrylamide gel electrophoresis; TXA_2, thromboxane A_2.

0006-291X/99 $30.00

(GP) VI was prepared from serum of a patient with GP VI-deficiency as described previously (24). Convulxin was isolated from *Crotalus durissus terrificus* venom as described previously (25). Activating transcription factor-2 (ATF-2) was expressed as His-tagged protein in *E. coli* and purified with Ni^+-NTA resin (Qiagen, Studio City, CA) as described previously (26). Recombinant human TPO (4) was kindly provided by Kirin Brewery Co. (Maebashi, Japan). A p38 inhibitor, SB203580, and its inactive analogue, SKF106978, were gifts from Dr. J. C. Lee (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) (27). Prostaglandin E_1 and 9, 11-epithio-11, 12-methano-thromboxane A_2 (STA_2) were kind gifts from Ono Pharmaceutical Co. (Osaka, Japan). Arg-Gly-Asp-Ser (RGDS), apyrase, epinephrine, ADP, and phorbol 12, 13-dibutyrate (PDBu) were obtained from Sigma. ^{32}Pi (8500-9120 Ci/mmol, 150 mCi/ml) and [γ-^{32}P]ATP (3000 Ci/mmol, 10 mCi/ml) were provided by DuPont NEN. The thrombin receptor agonist peptide SFFLRN (TRAP) was purchased from Phoenix Pharmaceuticals, Inc. (Mountain View, CA). All other reagents were obtained as described previously (28).

Preparation and stimulation of platelets. After informed consent was obtained, venous blood was collected from healthy adult donors. Anti-coagulation of blood and preparation of platelet-rich plasma were performed as described previously (28). Platelet-rich plasma was incubated with 1 mM aspirin for 30 min at 37°C. Gel-filtered platelets were then prepared as described previously (6) at a final concentration of 1×10^9 cells/ml in Hepes buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, 1 mg/ml bovine serum albumin, 3.3 mM NaH_2PO_4, and 20 mM Hepes, pH 7.4), containing 1 unit/ml apyrase and 500 μM RGDS. For ^{32}Pi-labeling of platelets, platelets were incubated with 0.5 mCi/ml ^{32}Pi in Hepes buffer without NaH_2PO_4 for 1 h at 37°C before gel-filtration. Gel-filtered platelets were stimulated by agonists with gentle agitation for appropriate periods at 37°C. In some experiments, gel-filtered platelets were preincubated with 0.1% (v/v) Me_2SO, 10 μM SB203580, or 10 μM SKF106978 for 10 min at 37°C, and then treated with buffer or with TPO for 3 min at 37°C before stimulation.

Immunoprecipitation and immunoblotting. Gel-filtered platelets were stimulated by various agonists with gentle agitation at 37°C and lysed in an equal volume of 2 × RIPA buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 10 mM EDTA, 2% Triton X-100, 0.2% SDS, 2% sodium deoxycholate, 80 mM β-glycerophosphate, 80 mM p-nitrophenylphosphate, 2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 40 μg/ml leupeptin, and 40 μg/ml aprotinin) to stop reaction. p38 was purified by immunoprecipitation from lysates with anti-p38 antibody as described previously (6). Immunoprecipitates from 2.6×10^8 cells/lane were subjected to 10% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE) and transferred electrophoretically to nitrocellulose membranes (Bio-Rad, Hercules, CA) with a semidry blotter. The membranes were treated for an immunoblot assay using the anti-phosphotyrosine monoclonal antibody 4G10 or anti-p38 antibody with the ECL chemiluminescence reaction (Amersham International plc, Little Chalfont, U.K.) as described previously (28).

In vitro kinase assay. p38 was isolated from unstimulated or stimulated platelets by immunoprecipitation as described above. Kinase activity of p38 was determined by an *in vitro* kinase assay with recombinant ATF-2 as described previously (29). Briefly, immunoprecipitated p38 was washed three times with RIPA buffer and two times with kinase buffer (25 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, and 0.1 mM Na_3VO_4), and then incubated for 30 min at 30°C with 3 μg of ATF-2 in 30 μl of kinase buffer including 1 μCi of [γ-^{32}P]ATP and 50 μM of ATP. Reactions were stopped by addition of 30 μl of 2 × SDS sample buffer. The samples were boiled for 3 min and subjected to 10% (w/v) SDS-PAGE followed by autoradiography.

Phosphorylation of Hsp27 and $cPLA_2$. ^{32}Pi-labeled platelets were stimulated and lysed in RIPA buffer as described above. Hsp27 and $cPLA_2$ were immunoprecipitated from lysates with anti-Hsp27 antibody and anti-$cPLA_2$ antibody, respectively, as described previously (6). Immunoprecipitates from 2.6×10^8 cells/lane were subjected to 12.5% (w/v) SDS-PAGE for Hsp27 or 7.5% (w/v) SDS-PAGE for $cPLA_2$, followed by autoradiography.

Densitometric analysis. The densities of protein bands on scanned images of immunoblot and autoradiogram were analyzed with the public domain NIH Image program (written by Wayne Rasband at the National Institute of Health).

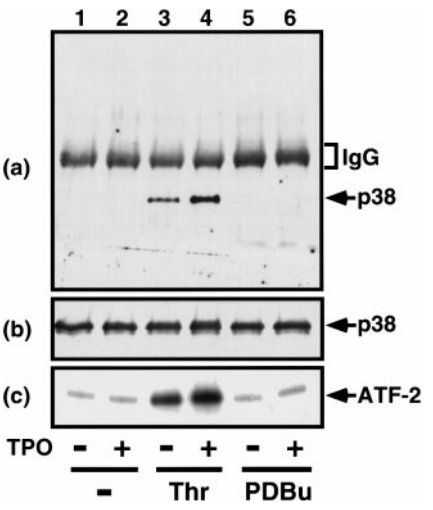

FIG. 1. Effects of TPO on tyrosine phosphorylation and activation of p38 induced by thrombin. Gel-filtered platelets pretreated with buffer alone (lanes 1, 3, and 5) or 50 ng/ml of TPO (lanes 2, 4, and 6) for 3 min were unstimulated (lanes 1 and 2) or stimulated with 0.1 unit/ml of thrombin (lanes 3 and 4) or 1 μM of PDBu (lanes 5 and 6) for 1 min under mild agitation, and were lysed to stop reaction. p38 was immunoprecipitated from the lysates and subjected to SDS-PAGE, followed by immunoblot analysis with anti-phosphotyrosine antibody (a) and with anti-p38 antibody (b). Kinase activity of p38 was determined with an *in vitro* kinase assay using ATF-2 as an exogenous substrate. Phosphorylation of ATF-2 was detected by autoradiography (c).

RESULTS

TPO enhances tyrosine phosphorylation and kinase activity of p38 stimulated by thrombin. We first studied tyrosine phosphorylation of p38 in platelets stimulated by TPO, thrombin, and the phorbol ester PDBu, or combinations of them. If not mentioned, gel-filtered platelets were pretreated with aspirin, apyrase, and RGDS to avoid the secondary effects of synthesized thromboxane A_2 (TXA_2), released ADP, and integrin $\alpha_{IIb}\beta_3$-mediated aggregation following platelet stimulation. As shown in Fig. 1a, in resting platelets, p38 was not phosphorylated on tyrosine. Thrombin stimulation induced tyrosine phosphorylation of p38, whereas TPO alone did not induce it. Interestingly, pretreatment of platelets with TPO distinctly enhanced the thrombin-induced tyrosine phosphorylation of p38. In contrast, PDBu did not stimulate p38 tyrosine phosphorylation irrespective of TPO pretreatment. We next examined kinase activity of p38 immunoprecipitated under the

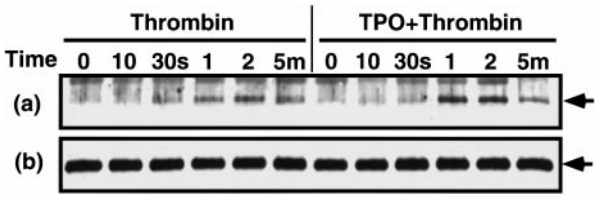

FIG. 2. Effects of TPO on time-dependent tyrosine phosphorylation of p38 induced by thrombin. Gel-filtered platelets pretreated with buffer alone or 50 ng/ml of TPO for 3 min were stimulated with 0.1 unit/ml of thrombin for the indicated periods under mild agitation, and were lysed to stop reaction. p38 was immunoprecipitated from the lysates and subjected to SDS-PAGE, followed by immunoblotting with anti-phosphotyrosine antibody (a) and with anti-p38 antibody (b). Arrows indicate the position of p38.

same conditions as described above by an in vitro kinase assay with ATF-2 as an exogenous substrate. Thrombin, but not TPO or PDBu, stimulated activity of p38, while TPO pretreatment potentiated the p38 activation induced by thrombin (Fig. 1c). Densitometric analysis revealed that upon thrombin stimulation, fold enhancement by TPO pretreatment in the tyrosine phosphorylation and the kinase activity of p38 was 1.87 ± 0.25 and 1.76 ± 0.13 (means ± SE, n = 3), respectively. These data confirmed that the kinase activity of p38 increased in proportion to the extent of tyrosine phosphorylation of the kinase. When platelets were not pretreated with aspirin, we also obtained essentially similar results on tyrosine phosphorylation and activation of p38 (data not shown). We examined a time course of tyrosine phosphorylation of p38 in thrombin-stimulated platelets pretreated with or without TPO. Tyrosine phosphorylation of p38 occurred after 30 s of thrombin stimulation and peaked between 1 and 2 min (Fig. 2). TPO pretreatment enhanced the level of tyrosine phosphorylation of p38 in thrombin-stimulated platelets with a time course similar to that seen with thrombin stimulation alone (Fig. 2). TPO by itself hardly induced tyrosine phosphorylation of p38 until 30 min (data not shown).

TPO enhances tyrosine phosphorylation of p38 stimulated by various agonists. We next examined the TPO effect on tyrosine phosphorylation of p38 in platelets stimulated by following agonists; TRAP, the TXA_2 analogue STA_2, collagen, convulxin, the $F(ab')_2$ fragment of anti-GP VI IgG, ADP, and epinephrine (Fig. 3). Convulxin (25, 30), a tropical rattle snake venom, and the $F(ab')_2$ fragment of anti-GP VI IgG (24) activate platelets by cross-linking of GP VI. TRAP, STA_2, collagen, convulxin, and the $F(ab')_2$ fragment of anti-GP VI IgG induced tyrosine phosphorylation of p38. Pretreatment of platelets with TPO markedly potentiated the p38 phosphorylation stimulated by these agonists (Fig. 3A and 3B). ADP, epinephrine, or their combination slightly induced tyrosine phosphorylation of p38. TPO pretreatment also enhanced the tyrosine phosphorylation of p38 stimulated by ADP, epinephrine, or their combination (Fig. 3C).

TPO enhances p38-dependent phosphorylation of Hsp27 and $cPLA_2$ induced by thrombin. We studied whether TPO affected the downstream pathways of p38. p38 phosphorylates and activates MAP-kinase-activated protein kinase-2, which in turn phosphorylates Hsp27 (31, 32). We then studied the effect of TPO on phosphorylation of Hsp27 in thrombin-stimulated platelets. Thrombin stimulation promoted Hsp27 phosphorylation, while TPO treatment by itself had no effect on it. However, TPO pretreatment further enhanced the Hsp27 phosphorylation by thrombin (Fig. 4A). To confirm whether p38 is involved in the Hsp27 phosphorylation, we used a selective inhibitor for p38, SB203580 (27), and an inactive analogue of it, SKF106978, as a control. 10 μM of SB203580 completely inhibited kinase activity of p38 in an in vitro kinase assay, if the inhibitor was added to the kinase buffer (data not shown). Preincubation of platelets with 10 μM of SB203580 but not SKF106978 com-

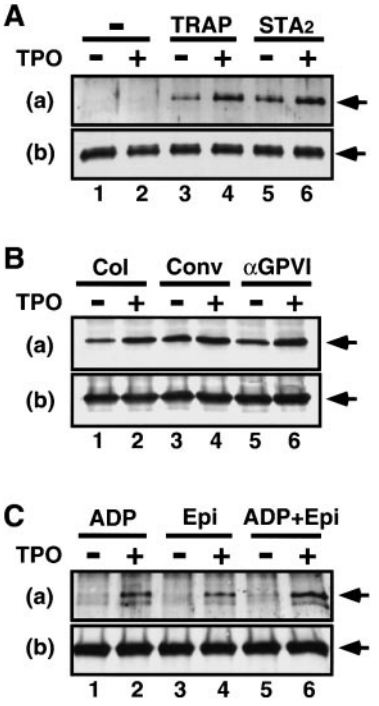

FIG. 3. Effects of TPO on tyrosine phosphorylation of p38 induced by various agonists. Gel-filtered platelets pretreated with buffer alone (lanes 1, 3, and 5) or 50 ng/ml of TPO (lanes 2, 4, and 6) for 3 min were unstimulated (−) or stimulated with 10 μM TRAP, 0.5 μM STA_2, 10 μg/ml collagen (Col), 20 ng/ml convulxin (Conv), 150 μg/ml the $F(ab')_2$ of anti-GPVI IgG (αGPVI), 20 μM ADP, 20 μM epinephrine (Epi), or with a combination of 20 μM ADP and 20 μM epinephrine (ADP+Epi) for 1 min under mild agitation, and were lysed to stop reaction. p38 was immunoprecipitated from the lysates and subjected to SDS-PAGE, followed by immunoblotting with anti-phosphotyrosine antibody (a) and with anti-p38 antibody (b). Arrows indicate the position of p38.

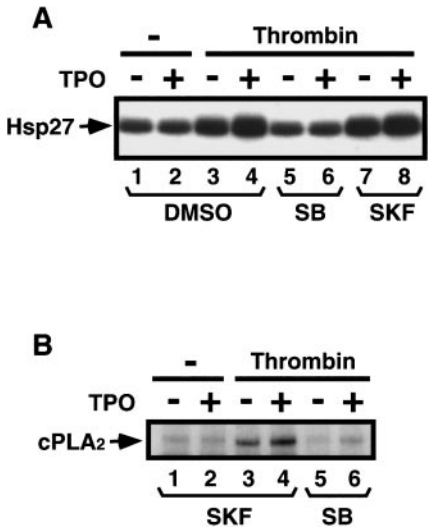

FIG. 4. Effects of TPO and the p38 inhibitor SB203580 on thrombin-induced phosphorylation of Hsp27 and $cPLA_2$. ^{32}Pi-labeled platelets were preincubated with 0.1% Me_2SO (DMSO), 10 μM SB203580 (SB), or 10 μM SKF106978 (SKF) for 10 min, treated with buffer alone or 50 ng/ml of TPO for 3 min, then stimulated with 0.1 unit/ml of thrombin for 1 min under mild agitation, and lysed to stop reaction. Hsp27 (A) and $cPLA_2$ (B) were immunoprecipitated from the lysates and subjected to SDS-PAGE, followed by autoradiography.

pletely blocked the thrombin-induced phosphorylation of Hsp27 irrespective of TPO treatment (Fig. 4A). The recent reports have shown that $cPLA_2$ is another candidate for substrates of p38 in platelets (22, 23). We then examined the effects of TPO and SB203580 on phosphorylation of $cPLA_2$ in thrombin-stimulated platelets (Fig. 4B). Again, TPO did not induce phosphorylation of $cPLA_2$ by itself but enhanced thrombin-induced phosphorylation of $cPLA_2$. Preincubation of platelets with 10 μM of SB203580 strongly inhibited the thrombin-induced phosphorylation of $cPLA_2$ irrespective of TPO treatment (Fig. 4B).

DISCUSSION

Previous studies have shown that p38 MAP kinase is involved in arachidonic acid release and aggregation in collagen- or TXA_2 analogue-stimulated platelets (21–23). We also observed p38 activation upon stimulation of various agonists such as thrombin, STA_2, TRAP, and collagen. Thrombin, TRAP, and STA_2 stimulate platelets through G protein-coupled receptors, whereas collagen stimulation is dependent on protein tyrosine phosphorylation including the Fc receptor γ chain and activation of the tyrosine kinase Syk (33). p38 was also activated by crosslinking of GP VI, one of collagen receptors, which is complexed with the Fc receptor γ chain (34–36). These data suggest common and important roles of p38 in platelet activation. On the other hand, the phorbol ester PDBu did not activate p38, indicating that p38 activation is independent of PKC. Interestingly, TPO markedly potentiated those receptor-mediated p38 activation by the agonists, whereas TPO by itself did not activate p38. Although TPO also potentiates platelet aggregation and release reactions (6–12), we can exclude the possibility that the TPO effect on p38 results secondarily from aggregation or release reactions for the following reasons. p38 was activated by various agonists except for PDBu in platelets pretreated with aspirin, apyrase, and RGDS, indicating that activation of p38 is independent of the arachidonic acid metabolism, released ADP, and integrin-mediated aggregation. Under these conditions, TPO enhanced p38 activation by these agonists. In contrast, PDBu elicits aggregation and release reaction without p38 activation. Furthermore, TPO still enhanced p38 activation in aspirinized platelets stimulated by weak agonists, ADP and epinephrine, although they do not induce release reactions in aspirinized platelets (37). Thus, we concluded that p38 activation and potentiation of it by TPO did not secondarily result from aggregation or release reaction. Therefore, the intracellular TPO signaling could directly synergize the pathway leading to p38 activation. The mechanism for the TPO potentiation effect remains to be revealed. Considering that TPO by itself did not stimulate either phosphorylation or activation of p38, it is tempting to speculate that TPO may reduce activity of protein phosphatases that down-regulate the p38 pathway or that TPO may induce intracellular translocation of some molecules in the p38 pathway to facilitate the p38 cascade activation by other agonists.

We next examined if TPO affected the downstream signals of p38. p38 phosphorylates and activates MAP-kinase-activated protein kinase-2, which in turn phosphorylates Hsp27 (31, 32), while p38 directly phosphorylates and activates $cPLA_2$ in some types of cells including platelets and neutrophils (22, 23, 29). TPO by itself did not induce phosphorylation of Hsp27 and $cPLA_2$, whereas TPO pretreatment augmented thrombin-induced phosphorylation of them. It was inhibited by the pyridinyl imidazole SB203580 (27), which is a specific inhibitor to p38 and has been used for identifying physiological roles of the p38 cascade in various cells (38), confirming that TPO potentiated phosphorylation of Hsp27 and $cPLA_2$ through the p38 pathway. Recently, Börsch-Haubold et al. has reported that SB203580 also directly inhibits cyclooxygenases and thromboxane synthase (39). However, we could exclude these effects of the inhibitor, since we used aspirinized platelets to block the arachidonic acid metabolism. Hsp27 seems to have protective activity against heat shock and other stressing agents (40). However, physiological functions of Hsp27 in platelets have been unknown. $cPLA_2$ is responsible for agonist-induced mobilization of arachidonic acid leading to TXA_2 synthesis

in platelets (41). Activity of $cPLA_2$ is regulated by its phosphorylation as well as cytosolic free calcium concentration (41). Thus, TPO could enhance TXA_2 synthesis via the p38-$cPLA_2$ pathway as the positive feedback of platelet activation.

Although TPO mRNA is expressed in several tissues, including the liver, kidneys, and bone marrow, the liver seems to be the major site of TPO production (42). Circulating TPO levels are thought to be directly regulated by the platelet and megakaryocyte mass (43) and markedly rise in patients with thrombocytopenia induced by radiation or chemotherapy. In such cases, the TPO priming effect on platelet activation could somewhat compensate bleeding tendency due to thrombocytopenia.

We have recently shown that TPO also potentiates agonist-induced activation of ERK1 and ERK2 in platelets (20). In contrast to p38, the ERK pathway is mainly dependent on PKC. TPO does not activate PKC or ERKs by itself but enhances activation of ERKs and MAP kinase/ERK kinase-1 and -2 (MEK1 and MEK2) in platelets (20). Taken together, it is now evident that the TPO signaling separately synergizes the two parallel MAP kinase pathways, the PKC-dependent ERK pathway and the PKC-independent p38 pathway.

ACKNOWLEDGMENTS

We thank Dr. J. C. Lee for SB203580 and SKF106978, Kirin Brewery Co. for TPO, and Ono Pharmaceutical Co. for STA_2 and prostaglandin E_1. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, Funds for Comprehensive Research on Aging and Health from the Ministry of Health and Welfare of Japan, and the Ryoichi Naito Foundation Grant for Medical Research. Y.E. is a Research Fellow of the Japan Society for the Promotion of Science.

REFERENCES

1. de Sauvage, F. J., Hass, P. E., Spencer, S. D., Malloy, B. E., Gurney, A. L., Spencer, S. A., Darbonne, W. C., Henzel, W. J., Wong, S. C., Kuang, W. J., Oles, K. J., Hultgren, B., Solberg, L. A., Goeddel, D. V., and Eaton, D. L. (1994) *Nature* **369,** 533–538.
2. Lok, S., Kaushansky, K., Holly, R. D., Kuijper, J. L., Lofton-Day, C. E., Oort, P. J., Grant, F. J., Heipel, M. D., Burkhead, S. K., Kramer, J. M., Bell, L. A., Sprecher, C. A., Blumberg, H., Johnson, R., Prunkard, D., Ching, A. F. T., Mathewes, S. L., Bailey, M. C., Forstrom, J. W., Buddle, M. M., Osborn, S. G., Evans, S. J., Sheppard, P. O., Presnell, S. R., O'Hara, P. J., Hagen, F. S., Roth, G. J., and Foster, D. C. (1994) *Nature* **369,** 565–568.
3. Bartley, T. D., Bogenberger, J., Hunt, P., Li, Y. S., Lu, H. S., Martin, F., Chang, M. S., Samal, B., Nichol, J. L., Swift, S., Johnson, M. J., Hsu, R. Y., Parker, V. P., Suggs, S., Skrine, J. D., Merewether, L. A., Clogston, C., Hsu, E., Hokom, M. M., Hornkohl, A., Choi, E., Pangelinan, M., Sun, Y., Mar, V., McNinch, J., Simonet, L., Jacobsen, F., Xie, C., Shutter, J., Chute, H., Basu, R., Selander, L., Trollinger, D., Sieu, L., Padilla, D., Trail, G., Elliott, G., Izumi, R., Covey, T., Crouse, J., Garcia, A., Xu, W., Castillo, J. D., Biron, J., Cole, S., Hu, M. C. T., Pacifici, R., Ponting, I., Saris, C., Wen, D., Yung, Y. P., Lin, H., and Bosselman, R. A. (1994) *Cell* **77,** 1117–1124.
4. Kato, T., Ogami, K., Shimada, Y., Iwamatsu, A., Sohma, Y., Akahori, H., Horie, K., Kokubo, A., Kudo, Y., Maeda, E., Kobayashi, K., Ohashi, H., Ozawa, T., Inoue, H., Kawamura, K., and Miyazaki, H. (1995) *J. Biochem.* **118,** 229–236.
5. Debili, N., Wendling, F., Cosman, D., Titeux, M., Florindo, C., Dusanter-Fourt, I., Schooley, K., Methia, N., Charon, M., Nador, R., Bettaieb, A., and Vainchenker, W. (1995) *Blood* **85,** 391–401.
6. Ezumi, Y., Takayama, H., and Okuma, M. (1995) *FEBS Lett.* **374,** 48–52.
7. Toombs, C. F., Young, C. H., Glaspy, J. A., and Varnum, B. C. (1995) *Thromb. Res.* **80,** 23–33.
8. Chen, J., Herceg-Harjacek, L., Groopman, J. E., and Grabarek, J. (1995) *Blood* **86,** 4054–4062.
9. Kojima, H., Hamazaki, Y., Nagata, Y., Todokoro, K., Nagasawa, T., and Abe, T. (1995) *Thromb. Haemost.* **74,** 1541–1545.
10. Montrucchio, G., Brizzi, M. F., Calosso, G., Marengo, S., Pegoraro, L., and Camussi, G. (1996) *Blood* **87,** 2762–2768.
11. Rodríguez-Liñares, B., and Watson, S. P. (1996) *Biochem. J.* **316,** 93–98.
12. Oda, A., Miyakawa, Y., Druker, B. J., Ozaki, K., Yabusaki, K., Shirasawa, Y., Handa, M., Kato, T., Miyazaki, H., Shimosaka, A., and Ikeda, Y. (1996) *Blood* **87,** 4664–4670.
13. Miyakawa, Y., Oda, A., Druker, B. J., Kato, T., Miyazaki, H., Handa, M., and Ikeda, Y. (1995) *Blood* **86,** 23–27.
14. Seger, R., and Krebs, E. G. (1995) *FASEB J.* **9,** 726–735.
15. Marshall, C. J. (1995) *Cell* **80,** 179–185.
16. Cano, E., and Mahadevan, L. C. (1995) *Trends Biochem. Sci.* **20,** 117–122.
17. Kyriakis, J. M., and Avruch, J. (1996) *J. Biol. Chem.* **271,** 24313–24316.
18. Papkoff, J., Chen, R. H., Blenis, J., and Forsman, J. (1994) *Mol. Cell. Biol.* **14,** 463–472.
19. Kramer, R. M., Roberts, E. F., Strifler, B. A., and Johnstone, E. M. (1995) *J. Biol. Chem.* **270,** 27395–27398.
20. Ezumi, Y., Uchiyama, T., and Takayama, H. (1998) *Eur. J. Biochem.* **258,** 976–985.
21. Saklatvala, J., Rawlinson, L., Waller, R. J., Sarsfield, S., Lee, J. C., Morton, L. F., Barnes, M. J., and Farndale, R. W. (1996) *J. Biol. Chem.* **271,** 6586–6589.
22. Kramer, R. M., Roberts, E. F., Um, S. L., Börsch-Haubold, A. G., Watson, S. P., Fisher, M. J., and Jakubowski, J. A. (1996) *J. Biol. Chem.* **271,** 27723–27729.
23. Börsch-Haubold, A. G., Kramer, R. M., and Watson, S. P. (1997) *Eur. J. Biochem.* **245,** 751–759.
24. Ichinohe, T., Takayama, H., Ezumi, Y., Yanagi, S., Yamamura, H., and Okuma, M. (1995) *J. Biol. Chem.* **270,** 28029–28036.
25. Polgár, J., Clemetson, J. M., Kehrel, B. E., Wiedemann, M., Magnenat, E. M., Wells, T. N. C., and Clemetson, K. J. (1997) *J. Biol. Chem.* **272,** 13576–13583.
26. Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E., and Hagiwara, M. (1996) *J. Biol. Chem.* **271,** 13675–13679.
27. Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., Strickler, J. E., McLaughlin, M. M., Siemens, I. R., Fisher, S. M., Livi, G. P., White, J. R., Adams, J. L., and Young, P. R. (1994) *Nature* **372,** 739–746.
28. Ezumi, Y., Takayama, H., and Okuma, M. (1995) *J. Biol. Chem.* **270,** 11927–11934.
29. Waterman, W. H., Molski, T. F., Huang, C. K., Adams, J. L., and Sha'afi, R. I. (1996) *Biochem. J.* **319,** 17–20.

30. Jandrot-Perrus, M., Lagrue, A. H., Okuma, M., and Bon, C. (1997) *J. Biol. Chem.* **272,** 27035–27041.
31. Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A. R. (1994) *Cell* **78,** 1027–1037.
32. Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., and Saklatvala, J. (1994) *Cell* **78,** 1039–1049.
33. Poole, A., Gibbins, J. M., Turner, M., van Vugt, M. J., van de Winkel, J. G. J., Saito, T., Tybulewicz, V. L. J., and Watson, S. P. (1997) *EMBO J.* **16,** 2333–2341.
34. Gibbins, J. M., Okuma, M., Farndale, R., Barnes, M., and Watson, S. P. (1997) *FEBS Lett.* **413,** 255–259.
35. Tsuji, M., Ezumi, Y., Arai, M., and Takayama, H. (1997) *J. Biol. Chem.* **272,** 23528–23531.
36. Ezumi, Y., Shindoh, K., Tsuji, M., and Takayama, H. (1998) *J. Exp. Med.* **188,** 267–276.
37. Mustard, J. F., Perry, D. W., Kinlough-Rathbone, R. L., and Packham, M. A. (1975) *Am. J. Physiol.* **228,** 1757–1765.
38. Cohen, P. (1997) *Trends Cell Biol.* **7,** 353–361.
39. Börsch-Haubold, A. G., Pasquet, S., and Watson, S. P. (1998) *J. Biol. Chem.* **273,** 28766–28772.
40. Arrigo, A. P., and Landry, J. (1994) *in* Heat Shock Proteins: Structure, Function, and Regulation (Morimoto, R. I., Tissieres, A., and Georgopoulos, C., Eds.), pp. 335–375, Cold Spring Harbor Press, New York.
41. Kramer, R. M., Roberts, E. F., Manetta, J. V., Hyslop, P. A., and Jakubowski, J. A. (1993) *J. Biol. Chem.* **268,** 26796–26804.
42. Qian, S., Fu, F., Li, W., Chen, Q., and de Sauvage, F. J. (1998) *Blood* **92,** 2189–2191.
43. Hou, M., Andersson, P. O., Stockelberg, D., Mellqvist, U. H., Ridell, B., and Wadenvik, H. (1998) *Br. J. Haematol. 101,* 420–424.