The Roles of Phosphatidylinositol 3-Kinase and Protein Kinase Cζ for Thrombopoietin-induced Mitogen-activated Protein Kinase Activation in Primary Murine Megakaryocytes*

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Thrombopoietin (TPO) stimulates a network of intracellular signaling pathways that displays extensive cross-talk. We have demonstrated previously that the ERK/mitogen-activated protein kinase pathway is important for TPO-induced endomitosis in primary megakaryocytes (MKs). One known pathway by which TPO induces ERK activation is through the association of Shc with the penultimate phosphotyrosine within the TPO receptor; however, several investigators found that the membrane-proximal half of the cytoplasmic domain of Mpl is sufficient to activate ERK in vitro and support base-line megakaryopoiesis in vivo. Using BaF3 cells expressing a truncated Mpl (T69Mpl) as a tool to identify non-Shc/Ras-dependent signaling pathways, we describe here novel mechanisms of TPO-induced ERK activation mediated, in part, by phosphoinositide 3-kinase (PI3K). Similar to cells expressing full-length receptor, PI3K was activated by its incorporation into a complex with IRS2 or Gab2. Furthermore, the MEK-phosphorylating activity of protein kinase Cζ (PKCζ) was also enhanced after TPO stimulation of T69Mpl, contributing to ERK activity. PKCζ and PI3K also contribute to TPO-induced ERK activation in MKs, confirming their physiological relevance. Like in BaF3 cells, a TPO-induced signaling complex containing p85PI3K is detectable in MKs expressing T61Mpl and is probably responsible for PI3K activation. These data demonstrate a novel role of PI3K and PKCζ in steady-state megakaryopoiesis.

Binding of TPO1 to its receptor, the product of the proto-oncogene c-mpl, activates a wide variety of signaling molecules and pathways. As for other cytokine systems, it is becoming clear that the response to TPO is characterized by networks of multiple branching and converging signaling pathways, which display extensive cross-talk. As such, blockade of one signaling pathway can be compensated by alternate pathways. This may partially explain relatively mild hematopoietic phenotypes of mice in which supposedly critical signaling pathways are disrupted by homologous recombination (1–3). We demonstrated previously that the ERK/MAPK pathway is activated in response to TPO in both a BaF3 cell line engineered to express full-length Mpl (BaF3/Mpl) and in primary MKs, and plays an important role in MK endomitosis (4). Consistent with our results, MKs from mice engineered to express only a truncated Mpl receptor missing 60 residues from the COOH terminus of the cytoplasmic domain (T61 or Δ60 mice) display a reduced capacity to activate ERK and have significantly decreased endomitotic capability after TPO administration in vivo (3). The classic pathway of ERK activation is via growth factor-induced Shc phosphorylation followed by its association with Grb2 (5), which then activates Sos, a nucleotide exchange factor for Ras (6). Consequently, ERK can be activated by Ras-GTP through Raf and MEK phosphorylation. Several groups have reported that Shc is strongly activated in response to TPO (7). Hence, Shc-dependent activation of Ras is likely to be an important mechanism of TPO-induced ERK activation. However, we also demonstrated that TPO stimulation of BaF3 expressing a truncated form of Mpl missing 52 residues from the COOH terminus of the cytoplasmic domain (BaF3/T69) could activate ERK without Shc phosphorylation (4) and support cell growth. Consistent with these results, platelets from T61 mice also retain some ability to activate ERK, independent of Shc (3). Therefore, Shc is not absolutely essential for ERK activation. Because the signals emanating from the full-length receptor are very diverse and redundant, studies of signaling from these truncated receptors allowed us to investigate only the minimally required set of signals for resting-state megakaryopoiesis. Furthermore, the pathways from the truncated Mpl to MAPK may be novel, as they are not mediated by the conventional Shc/Grb2/Sos/Ras pathway. Therefore, potentially new mechanisms of ERK activation have been explored in this study, including phosphoinositide 3-kinase (PI3K) and isoforms of protein kinase C (PKC).

Two types of PI3K have been shown to play important roles in cytokine-mediated signal transduction. Class IA PI3Ks, comprising p85 adapter and p110 catalytic subunits, are activated by cytokines and growth factors, whereas class IB PI3K (PI3KY), comprising p101 adapter and p110 catalytic subunits, is activated by heterotrimeric G protein-coupled receptors (reviewed in Ref. 8). A constitutively active form of class IA PI3K has been demonstrated to activate MAPK by stimulating Ras

The abbreviations used are: TPO, thrombopoietin; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase; IRS, insulin-like receptor substrate; PKC, protein kinase C; IL, interleukin; DN, dominant negative; PAGE, polyacrylamide gel electrophoresis; BIM, bisindolylmaleimide I; GST, glutathione S-transferase; MBP, myelin basic protein.
(9). Interference with the PI3K pathway, either by using pharmacological inhibitors (10–14) or expression of a dominant negative protein (9, 12) also blocks ERK activation, suggesting that PI3K is necessary for ERK activation in these systems. We have shown previously that TPO-induced PI3K activation is dependent on the recruitment of the active enzyme into signaling complexes containing Gab1/IRS docking proteins (15). Consistent with our results, a docking protein, Gab1, has been implicated in PI3K and thus ERK activation in gp130 receptor signaling (16). However, in other systems, constitutively active forms of this class of PI3K are insufficient to activate ERKs (17, 18). In one report, wortmannin-sensitive ERK activation was mediated by the class IB PI3K, PI3Kγ (19), and the effect was dependent on its protein kinase, not lipid kinase activity (20). Furthermore, PI3K-induced ERK activation has been shown to depend on both cell type and signal intensity; ERK activation depends on PI3K only at low signaling intensities (11). Therefore, several questions remain to be explained: how truncated Mpl receptors activate ERK, whether PI3K is activated by the truncated receptor in response to TPO, how it is activated, and whether it plays a role in ERK activation.

PKC is an expanding family of serine-threonine kinases, comprising numerous isoforms, which display varied patterns of tissue distribution and different physiological functions. Pharmacological agents modifying PKC that function in an isoform-specific manner have been generated, potentially providing clinically useful strategies to provide desirable therapeutic effects while minimizing adverse reactions. PKC was reported to be activated by TPO in U77/Mpl cells (21). However, the specific isoform utilization of PKCs and its contribution to ERK activation has not been reported in the TPO system. Several isoforms of PKCs have been shown to activate ERK (22). Interestingly, PKD1, a kinase dependent on PI3K, can activate atypical isoforms of PKCs (23–26). An atypical PKC isoform, PKCζ, has been implicated in Ras-independent ERK activation, either by direct phosphorylation of Raf1 (27) or of MEK (28), serving as a linker between PI3K and ERK pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—BaF3/Mpl cells were maintained in RPMI 1640 (Bio-Whittaker, Walkersville, MD) with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 2 mM/liter L-glutamine, 100 units/liter penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B (Bio-Whittaker, Walkersville, MD) supplemented with murine interleukin-3 (IL-3), obtained from the conditioned medium of engineered baby hamster kidney (BHK) cells. BaF3 cells expressing various truncation mutants of Mpl (29) and primary MK isolation from BDF1 mice by unit gravity sedimentation (4) were performed as described previously. 861I cells were a gift of Dr. Frederick de Sauvage (Genentech Inc., San Francisco, CA). 861I cells were isolated by a procedure similar to that for the wild type MKs, except that 10% fetal bovine serum was added to initial bone marrow cultures because these MKs developed poorly in the presence of TPO alone. At 48 h after culture initiation, nonadherent cells were transferred to a new flask because of excessive growth of adherent macrophages in serum-containing media. An MTT assay for cellular proliferation/survival in response to TPO was performed as reported previously (4).

Immunoprecipitation and Western Blot Analysis—Anti-Ras2, anti-Pi3K (p85 subunit), and anti-phosphotyrosine (4G10) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Dr. Toshio Hirano (Osaka, Japan) generously provided rabbit anti-Gab2 antibodies. Anti-phospho-Akt (Ser473) antibody and anti-phospho-ERK were obtained from New England Biolabs (Beverly, MA). All chemical inhibitors were purchased from Calbiochem (La Jolla, CA).

BaF3 cells and MKs were deprived of serum and cytokines for 14 and 6 h, respectively, before stimulation with 14 ng/ml murine TPO for 10 min, washed once with ice-cold phosphate-buffered saline, and lysed in a buffer containing 0.5% Nonidet P-40 as reported previously (15). The protein concentration of lysates was measured by Protein/DC assay (Bio-Rad). Specific proteins were immunoprecipitated from cell lysates by overnight incubation at 4°C with the indicated antibodies. Protein A/G-conjugated agarose beads (Santa Cruz) were then added and incubated for an additional 2 h at 4°C. The pelleted beads were then washed three times with lysis buffer, resuspended in gel electrophoresis loading buffer, and heated at 90°C for 5 min. The immunoprecipitates were subjected to SDS-PAGE and Western blot analysis. Bands were scanned and quantified by the ImageQuant software. The PI3K activity assay was performed as described previously (15).

Dominant Negative (DN) Experiments—A DN p85 Pi3K cDNA in Sreks was a gift from Dr. Wataru Ogawa (Kobe University School of Medicine, Kobe, Japan). A DN SHP2 construct in a eukaryotic expression vector, pCAGGS, was a generous gift from Dr. Hiroshi Maegawa (Shiga University, Kyoto, Japan). To generate cell lines, 100 μg of DN plasmids were co-transfected with 10 μg of pMX-puro (a gift from Dr. Toshio Kitamura) into BaF3/T69 cells by electroporation. A control culture was transfected with pMX-puro plasmid alone. Puromycin at a final concentration of 1 μg/ml was added 24 h after transfection. Expression of the dominant negative protein was determined by Western blot analysis of whole cell lysates.

Ras Assay (29)—Cells were lysed with MLB buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1% Noni P-40, 10% glycerol, 25 mM NaF, 10 mM MgCl2, 1 mM EDTA, 1 mM NaVO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin A, 1 mM NaVO4, and sonicated for 20 s. Five hundred μg of protein was pre-cleared with 1 μg of rabbit IgG and 20 μl of protein A agarose beads and immunoprecipitated overnight with 3 μg of anti-PKCζ. Forty μl of protein A-agarose beads were added and incubated for another 2 h. Beads were then washed two times with the high salt buffer (500 mM Tris, pH 7.4, 500 mM NaCl, 1% Nonidet P-40), three times with lysis buffer, and two times with kinase buffer (25 mM HEPES, pH 7.4, 25 mM MgCl2, 20 mM β-glycerophosphate, 20 mM α-nitrophosphate, 20 mM NaVO4, 2 mM dithiothreitol). One hundred of the last wash was collected for Western blot analysis of PKCζ to assess protein levels in the immune complexes. The kinase buffer was then added with 0.02 mM ATP, 5 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech), and either 5 μg of recombinant MEK1 (Santa Cruz, CA) or 16 μg of myelin basic protein (MBP) in a total volume of 25 μl. The kinase reaction was performed at 25°C for 1 h, stopped by adding 20 μl of Western loading buffer and boiling for 5 min, and subjected to SDS-PAGE. The gel was then dried and exposed to film, and the intensity of bands was quantitated by PhosphorImaging.

RESULTS

PI3K Is Activated in Response to TPO in BaF3/T69 Cells and Supports Cellular Proliferation—In searching for pathways responsible for Shc-independent ERK activation, the PI3K pathway was explored. As shown in Fig. 1A, TPO stimulation of BaF3/T69 clearly induced ERK phosphorylation, but to a lower extent than that seen in BaF3/Mpl cells. Using phosphorylation of Akt as a marker of PI3K activity, BaF3/T69 cells were found to retain the ability to activate PI3K in response to TPO, although weakly compared with that seen in BaF3/Mpl.

Our group has shown that PI3K is essential for cellular proliferation in BaF3/Mpl and primary MK (31). As TPO-induced Akt activation is much weaker in BaF3/T69 cells than in cells with full-length Mpl, its physiological relevance needs to be determined. BaF3/T69 cells proliferated in response to TPO, and concentrations of TPO was assessed by an MTT assay in the presence or absence of a specific PI3K inhibitor, Ly 294002. This inhibitor significantly decreased TPO-induced BaF3/T69 survival/proliferation in a dose-dependent manner (Fig. 1B). At 16 μM final concentration, there were almost no viable cells remaining in culture. Therefore, despite modest activation, PI3K plays an important role in proliferation of BaF3/T69 cells.
The Carboxyl-terminal Domain of Mpl Is Important for Gab2/SHP2/PI3K Complex Formation, but Not for IRS2/PI3K Association—Our previous studies have shown that the major mechanism of TPO-induced PI3K activation was via recruiting active enzyme into Gab- or IRS-containing signaling complexes (15). To expand upon our Western blot analyses, PI3K activity associated with Gab2- and IRS2-complexes was determined, comparing BaF3/Mpl and BaF3/T69 cells. Phosphoinositol was used as a substrate. After TPO stimulation, cell lysates were then subjected to Western blot analysis, probed with anti-phosphorylated ERKs (top panel) or anti-phosphorylated Akt (third panel). The blots were stripped and reprobed with anti-ERK2 and anti-Akt, respectively, for control of loading (second and fourth panels). Similar results were obtained from two experiments. B, BaF3/T69 cells were cultured in the presence of various concentrations of recombinant murine TPO. The cultures also contained Ly 294002 (dissolved in Me2SO) at 4 μM (×), 8 μM (△), or 16 μM (□) final concentrations, whereas the control cultures contained a similar volume of Me2SO (○). The number of living cells was determined after 36-h cultures using the MTT method. TPO-induced proliferation is presented as the percentage of maximal IL-3-induced proliferation. The abscissa is displayed on a logarithmic scale. The results represent the mean (± S.D.) of triplicate determinations of a single representative experiment. This experiment has been performed two times with similar results.

PI3K and PKCζ in TPO-induced MAPK Activation

FIG. 1. PI3K is activated in BaF3/T69 cells and plays a role in cellular proliferation. A, BaF3/Mpl or BaF3/T69 cells were serum- and growth factor-starved for 16 h before stimulation with 14 ng/ml TPO for 10 min. Cell lysates were then subjected to Western blot analysis, probed with anti-phosphorylated ERKs (top panel) or anti-phosphorylated Akt (third panel). The blots were stripped and reprobed with anti-ERK2 and anti-Akt, respectively, for control of loading (second and fourth panels). Similar results were obtained from two experiments. B, a diagram of Mpl truncation mutants used in this study. Only the intracytoplasmic domain of Mpl is shown. The locations of Tyr112, the major Shc binding site, and Box1 and Box2 motifs that bind JAK2 are indicated.

FIG. 2. IRS2/PI3K is a major signaling complex formed in BaF3/T69 cells. A, BaF3/Mpl or BaF3 T69 cells were starved of serum and growth factors for 16 h before stimulation with 14 ng/ml TPO for 10 min. Lysate was immunoprecipitated (IP) with either Gab2 or IRS2 antibodies as indicated. The PI3K activity in the immunoprecipitates was determined by an in vitro kinase assay (upper panel). One-tenth of the immunoprecipitates was reserved for Western blot analysis to assure equal amounts of Gab2 and IRS2 between stimulated and unstimulated lanes (lower panel). Similar results were obtained in two separate experiments. B, a diagram of Mpl truncation mutants used in this study. Only the intracytoplasmic domain of Mpl is shown. The locations of Tyr112, the major Shc binding site, and Box1 and Box2 motifs that bind JAK2 are indicated. C, BaF3 cells expressing various truncation forms of Mpl were starved and stimulated as described in A. Cell lysates were immunoprecipitated (IP) with a Gab2 antibody, subjected to Western blotting, and probed sequentially with anti-phosphotyrosine (top panel), anti-SHP2 (middle panel), and anti-Gab2 (lower panel). The positions of Gab2 and SHP2 bands on the blot are indicated by arrows and arrowheads, respectively. Similar results were obtained in two separate experiments.

(Fig. 2A, lower panel). PI3K activity was clearly detectable after TPO stimulation in Gab2 and IRS2 signaling complexes derived from both BaF3/Mpl and BaF3/T69 cells. In BaF3/Mpl, the Gab2-associated PI3K activity was more prominent than the IRS2-associated activity. In contrast, the TPO-induced Gab2-associated PI3K activity was significantly lower, and the
phosphorylation was unchanged by the presence of DNp85, expressing the DN p85 construct. However, TPO-induced JAK2 (pAkt) and 47 and 25% (pERK2) of that seen in control cells not inhibited both Akt and ERK activation in BaF3/T69 cells. In the results with the chemical inhibitor, DN p85 significantly protein for binding to Gab and IRS docking proteins. Similar to the catalytic subunit of PI3K, p110 (32). However, its SH2 negative construct of p85 PI3K was used. The dominant negative expression of Gab2 and SHP2 phosphorylation in BaF3 cells expressing full-length Mpl and various COOH-terminally truncated forms of Mpl (designated by T followed by the number of remaining cytoplasmic residues) is shown in a diagram (Fig. 2B). A truncation of Mpl that eliminates the two terminal tyrosine residues of Mpl (T98) significantly reduced Gab2 phosphorylation and SHP2 association (compare Mpl to T98; Fig. 2C, upper panel). Formation of the complex was further reduced in cells expressing shorter receptors, but as long as a receptor supported JAK2 phosphorylation (T98 through T53; Ref. 28) a modicum of Gab2 and SHP2 phosphorylation was seen.

A different result was obtained with the IRS2 complex. TPO-induced p85 PI3K association with IRS2 was not decreased in BaF3/T69, compared with that of BaF3/Mpl cells (data not shown). Consistent with the PI3K activity data, IRS2-containing complex formation was not found to be dependent on the terminal half of Mpl.

PI3K Is Necessary for ERK Activation in BaF3/Mpl and BaF3/T69 Cells—Specific PI3K inhibitors (Ly 294002 and wortmannin) were used to determine the roles of PI3K in ERK activation. In both BaF3/Mpl cells and BaF3/T69 cells, either Ly 294002 or wortmannin inhibited ERK phosphorylation in response to TPO (Fig. 3). Compared to cells without inhibitors, TPO-induced ERK1 and ERK2 phosphorylation in BaF3/Mpl cells was reduced by wortmannin at 100 nM final concentration to 62 and 32%, respectively (n = 2). As BaF3/T69 supported less ERK activation, wortmannin decreased ERK1 and ERK2 phosphorylation to 17 to 12% of control, respectively (n = 3). These data suggest that PI3K is critical for ERK activation by this truncated receptor.

PI3K and PKCζ in TPO-induced MAPK Activation

IRS2-containing complex became more prominent in BaF3/T69 cells.

To further explore the mechanisms of PI3K activation in BaF3/T69 cells, we studied the two PI3K-associated signaling complexes in response to TPO, Gab2/SHP2/PI3K and IRS2/PI3K. Gab2 and SHP2 phosphorylation in BaF3 cells expressing a truncation of Mpl that eliminates the two terminal tyrosine residues of Mpl (T98) significantly reduced Gab2 phosphorylation and SHP2 association (compare Mpl to T98; Fig. 2C, upper panel). Formation of the complex was further reduced in cells expressing shorter receptors, but as long as a receptor supported JAK2 phosphorylation (T98 through T53; Ref. 28) a modicum of Gab2 and SHP2 phosphorylation was seen.

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To assess the role of PI3K by a different means, a dominant negative construct of p85 PI3K was used. The dominant negative p85 contained a small deletion, rendering it unable to bind to the catalytic subunit of PI3K, p110 (32). However, its SH2 domains are intact, allowing it to compete with the wild type protein for binding to Gab and IRS docking proteins. Similar to the results with the chemical inhibitor, DN p85 significantly inhibited both Akt and ERK activation in BaF3/T69 cells. In two separate experiments, Akt and ERK phosphorylation were decreased by dominant negative expression to 57 and 55% (pAkt) and 47 and 25% (pERK2) of that seen in control cells not expressing the DN p85 construct. However, TPO-induced JAK2 phosphorylation was unchanged by the presence of DNp85, suggesting that the inhibition was specific (data not shown).

ERK Activation in BaF3/T69 Cells Is Independent of Ras and SHP2—SHP2 has been implicated in ERK phosphorylation and associates with Gab2 in response to TPO in BaF3/T69. In the lysophaspatidic acid and epidermal growth factor systems, binding of SHP2 to Gab1 is required for MAPK activation (37). A homologous SHP2 and Gab2 association in TPO system may function similarly. Therefore, the roles of SHP2 and Ras for ERK activation were investigated in BaF3/T69 cells.

First, Ras activation after TPO stimulation was assessed in BaF3/Mpl and BaF3/T69 cells. Cell lysates were incubated with the Ras binding domain of Raf1 coupled to Sepharose beads to pull down the active (GTP-bound) fraction of Ras. The precipitates were then washed, subjected to Western blot analysis, and probed with a pan-Ras antibody. Separate Western blotting was also performed on aliquots of whole cell lysate to assure equal amounts of total Ras (Total Ras) were present in stimulated and unstimulated conditions. Similar results were obtained from two separate experiments. B, BaF3/T69 cells were co-transfected with DN SHP2 and a plasmid containing a puromycin-selectable marker. Stable clones were obtained and tested for expression by Western blot analysis. One clone expressed only wild type SHP2 (70 kDa, WT), and another clone expressed the dominant negative form (24 kDa, DN) and were chosen for further study. C, phospho-ERKs (pERKs) were detected by Western blot after 10 min stimulation with 14 ng/ml TPO stimulation. ERK phosphorylation was not reduced in DN SHP2-expressing clone. To verify that the construct was active, the lysates were also immunoprecipitated with anti-SHP2 and probed sequentially for phosphotyrosine and SHP2. The positions of SHP2 bands on the blots are indicated by arrows. These experiments were performed twice per transfection. Similar results were found from two separate transfections.
By Ras, it is less likely that SHP2 induces ERK activation in BaF3/T69 cells.

To more directly investigate the role of SHP2 in ERK activation in BaF3/T69 cells, a dominant negative SHP2 construct was used (38). BaF3/T69 cells were stably transfected with a dominant negative SHP2 expression plasmid. Clones that highly expressed DN SHP2 and those that did not express the protein were selected for further study (Fig. 4B). Endogenous SHP2 phosphorylation after TPO stimulation was markedly inhibited in DN cells (Fig. 4C, second panel). As shown in the top panel of Fig. 4C, the DN SHP2 had no effect on ERK activation in TPO-stimulated BaF3/T69 cells.

PKCζ contributes to TPO-induced ERK activation in BaF3/T69 cells—Atypical isoforms of PKC, particularly PKCζ, have been implicated in Ras independent ERK activation in other cell systems (30, 39, 40). Therefore, we considered PKCζ a potential candidate for mediating Ras-independent ERK activation in BaF3/T69 cells. The function of PKC isoforms was initially evaluated using two pharmacological inhibitors of PKCs, low concentrations of bisindolylmaleimide I (BIM), and Ro 31-8220. At low concentrations BIM is an inhibitor of the conventional and novel isoforms of PKC, displaying only minor effects on atypical isoforms of PKCs. In contrast, Ro 31-8220 is a relatively specific inhibitor of atypical isoforms of the kinase. TPO-induced ERK activation in BaF3/T69 cells was not affected by BIM (Fig. 5A), although PMA-induced activation of ERK was blocked (indicating that the inhibitor was active), suggesting that conventional and novel isoforms of PKCs are not essential for ERK activation by T69Mpl. In contrast, Ro 31-8220 significantly decreased TPO-induced ERK activation in BaF3/T69 cells (Fig. 5B). Serum- and IL-3-induced ERK activation was not affected by Ro 31-8220, suggesting that the effect of this inhibitor was specific. Combined with the data with BIM, this result suggests that atypical isoforms of PKC may play an important role in ERK activation.

To confirm the effect of the chemical inhibitors, a cell-permeable PKCζ pseudosubstrate peptide that binds to the active site of the enzyme blocking its function was employed. We found the PKCζ pseudosubstrate peptide markedly inhibited ERK activation in response to TPO in BaF3/T69 cells (Fig. 5C), although it did not affect STAT3 activation (indicating a lack of general toxicity), confirming an essential role for this PKC isoform in TPO-induced ERK activation.

PKCζ is activated in response to TPO in BaF3/T69 cells—We further investigated whether PKCζ is activated in response to TPO in BaF3/T69 cells. PKCζ kinase assays, using recombinant MEK1 or MBP as a substrate, were performed on PKCζ immune complexes. The results from three to four experiments were averaged and a representative autoradiography is shown in Fig. 6. TPO stimulation for 5 min doubled the in vitro MEK1-phosphorylating activity of PKCζ (Fig. 6A). In addition, TPO stimulation for 1 min significantly enhanced the MBP-phosphorylating activity of PKCζ by ~60% (Fig. 6B, p = 0.03 by paired t test). To assess the effects of PI3K on PKCζ activation, Ly 294002 was used before stimulation and lysates were subjected to in vitro kinase assays using MBP as a substrate. This activation was modestly inhibited by Ly 294002; however, the significance of this inhibition is unclear. In four separate experiments, Ly 294002 decreased TPO-induced PKCζ activity in BaF3/T69 cells but the magnitude of reduction did not reach statistical significance.

ERK activation in primary MKs is PKCζ-dependent—To verify the physiological relevance of these TPO-induced, Shc-independent ERK activation pathways in BaF3/T69 cells, we further investigated signaling pathways in primary MKs. As shown in Fig. 7A, Ro 31-8220 markedly inhibited TPO-induced ERK activation in MKs, but BIM had little or no effect (Fig. 7B), again suggesting an important role for atypical isoforms of PKC in TPO signal transduction. Interestingly, Akt phosphorylation in response to TPO was enhanced by Ro 31-8220, indicating that the effect of the inhibitor was not a result of general cellular toxicity, and that PKCζ was not upstream of PI3K. PKCζ may suppress PI3K activity as a feedback mechanism. In contrast, the PI3K inhibitor did not affect ERK activation in primary MKs, despite complete abrogation of Akt phosphorylation. This difference from our results in BaF3 cells could be the result of a prominent effect of the Sho/Grb/Sos pathway in MKs, obscuring the function of PI3K on ERK activation. To explore whether PI3K contributes to ERK activation in MKs, we tested cells from T61 mice, where the Sho/Grb-Sos pathway is eliminated.

PI3K is activated by TPO in T61 (∼60%) MKs and contributes to ERK activation—Because MKs containing a full-length Mpl receptor can activate ERK through several pathways, blockade of only the PI3K pathway may be by-passed by the Sho-Grb-Sos pathway. Therefore, we further investigated signaling path-
ways in T61 MKs, derived from mice engineered to express a truncated Mpl receptor by knock-in technology (3). In vitro, MK number and size respond poorly to TPO (data not shown). Thus, only very limited amounts of protein could be obtained from T61 MKs, despite our attempts to expand MKs using fetal bovine serum, IL-11, and/or IL-6. Consequently, each experiment using T61 MKs was performed only twice, and the densitometer-assessed band intensities of all experiments are reported.

As shown in Fig. 8A, although reduced compared with normal MKs, TPO-induced ERK activation in MKs derived from T61 mice; in two separate experiments, ERK2 phosphorylation was enhanced 2.0- and 2.6-fold by TPO. Furthermore, Ly 294002 significantly inhibited ERK activation in TPO-stimulated T61 MKs (Fig. 8B); in two separate experiments, TPO-induced ERK activation in primary MKs was performed only twice, and the densitometer-assessed band intensities of all experiments are reported.

As shown in Fig. 8A, although reduced compared with normal MKs, TPO-induced ERK activation in MKs derived from T61 mice; in two separate experiments, ERK2 phosphorylation was enhanced 2.0- and 2.6-fold by TPO. Furthermore, Ly 294002 significantly inhibited ERK activation in TPO-stimulated T61 MKs (Fig. 8B); in two separate experiments, TPO-induced ERK2 phosphorylation was reduced by Ly 294002 to 57 and 11% of that seen without the inhibitor. SHP2 phosphorylation was enhanced by TPO stimulation by 4.5- and 8.2-fold (Fig. 9A) in T61 MKs in two experiments. Moreover, the complex of pp100/PI3K/SHP2 we detected previously in wild type MKs (15) was detectable after TPO stimulation, although very weakly. The pp100 band intensities (Fig. 9B) were increased 3.9- and 2.8-fold by TPO (Fig. 9B, upper panel). The SHP2-associated p85 bands (Fig. 9C, upper panel) were enhanced 10.3- and 12.6-fold after TPO stimulation. These data suggest that activation of ERK, SHP2, and PI3K does not absolutely require the terminal half of Mpl in primary MKs, and may contribute to base-line MK development.

**DISCUSSION**

The TPO receptor Mpl generates a wide variety of signals that impact upon cellular survival, proliferation, and differentiation. Based on the capacity of a truncated TPO receptor to support cellular growth in vitro and to maintain base-line platelet production in vivo, yet fail to stimulate a full repertoire of signals, it appears that several TPO-induced signaling pathways are superfluous, at least for steady-state thrombopoiesis. Moreover, several essential Mpl-induced signaling molecules can be activated by more than one pathway. For example, ERK is activated by both Shc-dependent and Shc-independent pathways (3, 4). In addition, our data have shown previously that PI3K can be activated by either Gab2 or IRS2 complex formation (15). Recent reports have illustrated the effects of disrupting one (or a few) Mpl signaling pathways in whole animals (2, 3). Interestingly, these mice display normal base-line MK development.

**FIG. 6.** PKCζ is activated in response to TPO in BaF3/T69 cells. BaF3/T69 cells were deprived of serum and growth factors for 16 h before stimulation with 14 ng/ml TPO for indicated time. Cell lysate was immunoprecipitated (IP) with anti-PKCζ antibody, or rabbit IgG negative control (IgG), and subjected to an in vitro kinase assay using MEK1 (A) or MBP (B) as substrates. The reactions were stopped by the addition of gel loading buffer and size-fractionated by SDS-PAGE. Dried gels were exposed to film as shown. Additionally, the bands were quantified by PhosphorImager and expressed as percentage of base line. Each bar is the average and S.D. from three separate experiments.

**FIG. 7.** Atypical isoforms of PKC contribute to ERK activation in primary MKs. MKs were starved of serum and growth factors for 6 h before stimulation with 14 ng/ml TPO for 10 min. A and B, Ro 31-8220 at 20 μM final concentration and/or Ly 294002 at 50 μM final concentration (A) or BIM at indicated concentrations (B), or an equal volume of Me2SO control were added 30 min before stimulation. Cell lysate was subjected to Western blot analysis and probed sequentially with anti-phosphorylated Akt and anti-phosphorylated ERKs (upper panels). Blots were stripped and re-probed with anti-ERK2 to assure equal loading (lower panel). Similar results were obtained from two separate experiments.
PI3K, is phosphorylated in response to TPO in BaF3/T69 cells, suggesting that the PI3K pathway can be activated by the truncated receptor. Consistent with this result, p85 associated with Gab2- and IRS2-containing signaling complexes and was activated in response to TPO. Similar to the results in BaF3 cells with the full-length receptor, PI3K activation was required for BaF3/T69 cell proliferation and survival as demonstrated by the use of the PI3K inhibitor Ly 294002 at concentrations just sufficient for Akt blockade, suggesting that this modest PI3K activation is physiologically important. Furthermore, we also demonstrate that class IA PI3K contributes TPO-induced ERK activation in BaF3/T69 cells using both pharmacological inhibitors and a dominant negative p85 construct.

To investigate the site within the Mpl receptor from which PI3K activation originates, BaF3 cells expressing various COOH-terminal truncation mutants of Mpl were tested for PI3K-containing complexes after TPO stimulation. Our group demonstrated previously that mutations of two terminal tyrosine residues, Tyr117 and Tyr112, of Mpl reduced, but did not abolish, TPO-induced BaF3 cell proliferation (28). In this study, deletion of these terminal two tyrosine residues, the T98 truncation, was still present in BaF3/T69, albeit reduced compared with BaF3/Mpl cells. This finding suggests that the Gab2/SHP2/PI3K complex contributes to PI3K activation in TPO-stimulated BaF3/Mpl cells, as TPO-induced Akt phosphorylation is significantly diminished in BaF3/T69 cells. In contrast to the Gab2-based PI3K complex, the IRS2/PI3K complex formed in BaF3/T69 was of similar intensity to that seen in BaF3/Mpl cells and the PI3K activity associated with this complex was also similar. Therefore, phosphorylation of IRS2 and Gab2 does not absolutely require phosphorytrosine docking sites or other sites within the distal 52 residues of c-Mpl.

FIG. 8. TPO-induced ERK activation in T61 MKs is dependent on PI3K. A, primary MKs from wild type (WT) or T61 mice were deprived of serum and growth factors for 6 h before stimulation with 14 ng/ml TPO for 10 min. Cell lysate was subjected to Western blot analysis and probed with anti-phosphorylated ERKs (upper panel). Blots were stripped and re-probed with anti-ERK2 for control of loading (lower panel). Similar results were obtained from two separate experiments. B, MKs from T61 mice were starved and stimulated as in A, but 50 μM Ly 294002 or an equal volume of MeSO were added 30 min before stimulation. Cell lysate was subjected to Western blot analysis and probed sequentially with anti-phosphorylated ERKs (upper panel) and anti-ERK2 for control of loading (lower panel). Similar results were obtained from two separate experiments.

FIG. 9. A TPO-induced, PI3K-containing multi-protein signaling complex is present in T61 MK. Primary MKs from T61 mice were deprived of serum and growth factors for 6 h before stimulation with 14 ng/ml TPO for 10 min. Lysate was then immunoprecipitated (IP) with anti-SHP2 antibody (A), anti-p85 antibody (B), or anti-SHP2 antibody (C), subjected to Western blot analysis, and probed sequentially with anti-phosphotyrosine or anti-p85 (upper panels) and anti-p85 or anti-SHP2 (lower panels). Similar results were obtained in two separate experiments. The bands from both experiments were scanned and quantified (see text).
presence or absence of TPO. Using either MEK1 or MBP as a substrate TPO significantly enhanced cellular PKCζ activity. Therefore, PKCζ may activate ERK, at least partly, by direct phosphorylation of the MAPK kinase, MEK, in cells. However, the phosphorylation of the MAPK kinase kinase, Raf1, by PKCζ has also been reported (27) and may contribute to this Ras-independent ERK activation. As PKCζ has previously been shown to be a target of the PI3K-dependent kinase, PDK1 (23, 25, 45), we hypothesized that the membrane proximal region of Mpl activates ERK through IRS2/Pi3K and PKCζ. Pretreatment of cell with Ly 294002, a PI3K inhibitor, decreased PKCζ phosphorylation, but is still able to activate ERKs via another (PKC activation in these MKs is not sufficient to support Akt phosphorylation. Alternatively, PI3K activation in T61 MKs (Fig. 8A) is possible caused by a deletion of a negative regulatory region of Mpl.

In primary MKs, a Tyr-phosphorylated protein that runs with a mass of ~100 kDa, termed pp100, can be co-immunoprecipitated with P85 PI3K (15). We postulated that pp100/SHP2/PI3K complex in T61 MKs (Fig. 8A). This novel Shc-independent TPO-induced ERK activation may play an important role in megakaryopoiesis.

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The Roles of Phosphatidylinositol 3-Kinase and Protein Kinase Cζ for Thrombopoietin-induced Mitogen-activated Protein Kinase Activation in Primary Murine Megakaryocytes

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