Thrombopoietin (TPO) is a recently characterized member of the hematopoietic growth factor family that serves as the primary regulator of megakaryocyte (MK) and platelet production. The hormone acts by binding to the Mpl receptor, the product of the cellular proto-oncogene c-mpl. Although many downstream signaling targets of TPO have been identified in cell lines, primary MKs, and platelets, the molecular mechanism(s) by which many of these molecules are activated remains uncertain. In this report we demonstrate that the TPO-induced activation of phosphoinositol 3-kinase (PI3K), a signaling intermediate vital for cellular survival and proliferation, occurs through its association with inducible signaling complexes in both BaF3 cells engineered to express Mpl (BaF3/Mpl) and in primary murine MKs. Although a direct association between PI3K and Mpl could not be demonstrated, we found that several proteins, including SHP2, Gab2, and IRS2, undergo phosphorylation and association in BaF3/Mpl cells in response to TPO stimulation, complexes that recruit and enhance the enzymatic activity of PI3K. To verify the physiological relevance of the complex, SHP2-Gab2 association was disrupted by overexpressing a dominant negative SHP2 construct. TPO-induced Akt phosphorylation was significantly decreased in transfected cells suggesting an important role of SHP2 in the complex to enhance PI3K activity. In primary murine MKs, TPO also induced phosphorylation of SHP2, its association with p85 and enhanced PI3K activity, but in contrast to the results in cell lines, neither Gab2 nor IRS2 are phosphorylated in MKs. Instead, a 100-kDa tyrosine-phosphorylated protein (pp100) co-immunoprecipitated with the regulatory subunit of PI3K. These findings support a model where PI3K activity is dependent on its recruitment to PI3K signaling complexes, implicating the existence of a scaffolding protein in primary MKs distinct from the known Gab and IRS proteins, and suggest that, in contrast to erythroid progenitor cells that employ Gab1 in PI3K signaling complexes, utilization of an alternate member of the Gab/IRS family could be responsible for specificity in TPO signaling.

The regulation of platelet production is a complex process. Maintained within relatively normal limits, both reduced and excessive platelet production leads to pathologic bleeding or thrombosis. Much is known of the cell biology of this process. Bone marrow megakaryocytes (MKs) are the immediate cellular precursors of blood platelets, and a hierarchy of stem cells, multipotent progenitors, and progenitors committed to the MK lineage are responsible for continuously renewing the marrow pool of MKs (1). Recently, the primary regulator of this developmental pathway, thrombopoietin (TPO), and its cellular receptor, the product of the proto-oncogene c-mpl, were molecularly cloned and characterized (2). In a number of cell culture systems TPO has been shown to affect all aspects of MK development, supporting the survival, proliferation, and differentiation of cells from the hematopoietic stem cell to the mature MK (3–7). In keeping with these in vitro findings, the genetic elimination of tpo or c-mpl leads to greatly reduced levels of hematopoietic stem cells, multipotent and committed progenitors, mature MKs, and blood platelets (8–10), indicating that TPO is a general regulator of hematopoiesis and the major humoral factor controlling MK and platelet production in vivo.

The intracellular signaling pathways utilized by c-mpl have been extensively studied by many groups of investigators. After stimulation with TPO the Mpl receptor is believed to change conformation resulting in a homodimeric receptor complex capable of supporting the transphosphorylation and activation of two tethered Janus kinases, JAK2 and TYK2 (11–14). However, only JAK2 appears to be essential for subsequent signaling events (15). In contrast to these reports, one series of experiments has called the importance of JAK2 activation into question (16). Nevertheless, once activated, Janus kinases phosphorylate a number of substrates, including the Mpl receptor itself (providing a docking site for SH2-containing proteins), the latent transcription factors signal transducers and activators of transcription (STAT) 3 and STAT5, and a number of adapter proteins including She and SHP2. Other signaling intermediates have also been found to be activated by TPO in various cell lines including mitogen-activated protein kinase (MAPK; see Refs. 17 and 18), protein kinase C (19, 20), and PI3K (16, 21, 22), molecules that play vital roles in MK development. For example, in previous studies we have shown that MAPK is activated in primary MK and is essential for nuclear endomitis (18), a characteristic feature of MK development, and that the protein kinase Ca isofrom is essential for proplatelet formation.

Thrombopoietin Induces Phosphoinositol 3-Kinase Activation through SHP2, Gab, and Insulin Receptor Substrate Proteins in BAF3 Cells and Primary Murine Megakaryocytes*
let formation (23), the process by which platelets develop from MK cytoplasm. Notably, TPO-induced MAPK activation has been shown to be both Src-dependent and Src-independent in both cell lines and platelets (24, 25). However, the Src-independent pathway of MAPK activation that follows TPO signaling remains undefined.

Much recent work has focused on the role of PI3K in supporting cell survival. One of the best studied mechanisms by which PI3K prevents programmed cell death is mediated by its activation of Akt, leading to phosphorylation and sequestration of the apoptosis-promoting Bcl family member Bad (26). Many investigators have reported that several hematopoietic cytokines, including interleukin (IL)-3 (26, 27), erythropoietin (EPO; see Refs. 28 and 29), and IL-6 (30) can activate PI3K and Akt. However, PI3K has also been shown to mediate several other cellular events, including proliferation (31, 32). In addition to its effects on Akt, PI3K can affect other signaling events, including the Ras/Raf/MEK/ERK and EGFR pathways, both of which can play roles in cellular proliferation.

The mechanism of PI3K activation has been extensively studied in several cytokine-responsive cell lines. For some receptors that induce PI3K activation, such as the EPO, platelet-derived growth factor, or Fli3 receptors, a 4-residue site of the form pYXXM or pYVAC (where pY is phosphotyrosine) has been described to bind directly the regulatory subunit of PI3K (33–35). Neither of these sites exists within the cytoplasmic domain of the Mpl receptor. However, in other receptors, complexes of p85 with scaffolding/adaptor proteins have been identified to induce PI3K activation by conformational changes in p85 and by bringing the catalytic subunit of PI3K, p110, to the cell membrane in close proximity to its phospholipid substrates (36). This adapter mechanism has also been studied in normal erythroid progenitor cells, in which Gab1 has been identified to play a scaffolding role in PI3K activation (37). However, although PI3K has been shown to be activated by TPO in several cell lines (21, 36, 38), its activation has never been reported in primary MKs, and the TPO-induced signaling pathways leading to SHP2 and PI3K are still to be determined in cells of this lineage. Therefore, in the present study the role of scaffolding/adaptor molecules in the TPO-induced activation of PI3K has been explored using both BaF3/Mpl cells and primary murine MKs.

Insulin receptor substrates (IRS) and their related proteins, Grb2-associated binders (Gab), are involved in many cytokine-signaling events (37, 39). Gab/IRS proteins have a pleckstrin homology domain responsible for recruitment to the plasma membrane and several protein-protein docking motifs that when phosphorylated allow the assembly of multiprotein signaling complexes. At least five IRS and three Gab adapters have been reported, but additional members are likely to be identified. Included among the signaling intermediates that associate with Gab/IRS proteins are the p85 subunits of PI3K, SHP2, Grb-2, and Crk. However, the role(s) of Gab/IRS proteins in megakaryopoiesis have not yet been described. As engagement of TPO by its receptor has been shown to activate a wide variety of signaling pathways, we tested whether one or more of these proteins is involved in TPO signaling in MKs. We found that several proteins, including SHP2, Gab2, and IRS2, undergo phosphorylation and association in BaF3/Mpl cells following exposure to TPO, complexes that recruit and enhance the enzymatic activity of PI3K. Furthermore, SHP2 was found to be important for full PI3K activation suggesting the role of Gab/IRS proteins in recruiting SHP2 and PI3K into the same complex. In primary murine MKs, TPO also induced phosphorylation of SHP2, its association with p85, and enhanced associated PI3K activity, but in contrast to the results in cell lines or in erythroid progenitor cells, none of the known Gab or IRS proteins were phosphorylated in MKs. Instead, a 100-kDa tyrosine-phosphorylated protein (p100) co-immunoprecipitated with the regulatory subunit of PI3K. These data provide further insights into the molecular mechanisms of megakaryopoiesis by identifying several molecules upstream of PI3K employed during TPO signaling.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Purified recombinant murine TPO was a generous gift of Dr. Akira Shimosaka (Kiris Pharmaceuticals, Tokyo, Japan), and human TPO was kindly provided by Dr. Donald Foster (ZymoGenetics, Inc., Seattle, WA). Anti-IRS1 and anti-Grb2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IRS2, anti-SHP2, anti-PI3K (p85 subunit), and anti-phospho-tyrosine (4G10) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). A rabbit polyclonal anti-c-Mpl antisera was the generous gift of Dr. Donald Foster (ZymoGenetics), and the rat AMM2 monoclonal antibody against murine c-Mpl was the kind gift of Dr. Takashi Kato (Kiris). Dr. Toshio Hirano (Osaka, Japan) generously provided rabbit anti-Gab1 and anti-Gab2 antibodies, and Dr. Larry Rohrschneider kindly provided the anti-Gab3 antibody. Anti-phospho-Akt (Ser473) antibody was obtained from New England Biolabs (Beverly, MA). Western blot chemiluminescence reagents were purchased from PerkinElmer Life Sciences, and all other reagents were purchased from Sigma.

**Cell Lines and Cell Culture Conditions—**The murine IL-3-dependent cell line BaF3 was engineered to express the murine Mpl receptor (BaF3/Mpl; see Ref. 40) and was maintained in RPMI 1640 medium (BioWhittaker, Walkersville, MD) with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT) and murine IL-3 (0.2% (v/v) conditioned medium from baby hamster kidney cells engineered to secrete IL-3). The human leukemic cell line UT-7/TPO (kindly provided by Dr. Norio Komatsu) was maintained in Iscove’s modified Dulbecco’s medium (IMDM, Sigma) with 10% fetal calf serum and 5 ng/ml human TPO. These cell lines were serum- and growth factor-deprived by incubation overnight in culture medium supplemented only with 0.5% bovine serum albumin (BSA). To obtain primary murine MKs, BDF-1 mice (Jackson Laboratories, Bar Harbor, ME) were subcutaneously injected with 2 μg of human TPO daily for 5 days, and bone marrow cells were obtained by flushing and were cultured for 3 days in IMDM supplemented with 1% Nutridoma SP (Roche Molecular Biochemicals) and 250 ng/ml human TPO. The cultured mature MKs were purified by unit gravity sedimentation on a discontinuous BSA density gradient as described previously (41). The purity of the collected cells was greater than 90% MKs, which were identified by acetylcholinesterase staining. MKs were starved in IMDM with 1% Nutridoma without cytokines for 7 h prior to signaling studies.

**Immunoprecipitation and Western Blot Analysis—**Serum- and cytokine-starved BaF3/Mpl cells and MKs were stimulated with 25 ng/ml murine TPO for 10 min, washed once with ice-cold phosphate-buffered saline (PBS), and lysed in a buffer composed of 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 100 mM NaF, and 0.5% Nonidet P-40. 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 Biotechnology) were then added and incubated for 2 additional hours at 4 °C. The pelleted beads were then washed 3 times with lysis buffer, resuspended in gel electrophoresis loading buffer, and heated to 90 °C for 5 min. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis, as described previously (11).

**Phosphoinositol 3-Kinase Assay—**BaF3/Mpl cells were incubated overnight in RPMI 1640 medium with 0.5% BSA and purified MKs in 1% Nutridoma in IMDM for 7 h. The starved cells were stimulated with 25 ng/ml murine TPO for 10 min, lysed, and immunoprecipitated as described above. Two hours after adding protein A or protein G-agarose beads, the immune complexes were washed three times with the lysis buffer, three times with 0.1 M Tris-HCl, pH 7.4, 5 mM LiCl, and 0.1 M sodium orthovanadate, and twice with TNE buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.1 mM sodium orthovanadate). After the final wash, 50 μl of TNE, 10 μl of 2 mg/ml sonicated phosphatidylinositol in 10 mM Tris-HCl with 1 mM EGTA, 10 μl of 0.1 M MgCl2, and 5 μl of ATP mix (0.88 mM ATP containing 30 μCi of [γ-32P]ATP and 20 mM MgCl2) were added sequentially. The kinase reaction mix was incubated at 37 °C for 10 min before stopping the reaction by adding 20 μl of 0.5% HCl. The radiolabeled lipid was then extracted...
with 160 μl of 1:1 CHCl₃:methanol and centrifuged for 10 min. Fifty μl of the lower organic phase was spotted on a silicon TLC plate (EM Science, Gibbstown, NJ) pretreated with 1% potassium oxalate and separated by chromatography in CHCl₃:methanol:H₂O:NH₄OH (120:94:22.6:4) for 3 h. The plates were dried and visualized by autoradiography and quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Dominant Negative SHP2 Experiments**—We obtained a dominant negative SHP2 (DN SHP2) construct consisting of the pair of SH2 domains of SHP2 (residues 1–216) in a eukaryotic expression vector, pCAGGS (a generous gift from Dr. Hiroshi Maegawa, Shiga, Japan). Ten μg of DN SHP2 plasmid was co-transfected with 1 μg of the pMX-puro plasmid (a gift from Dr. Toshio Kitamura) into BaF3/Mpl 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 probed with a monoclonal antibody to the N terminus of SHP2 (Transduction Laboratories, San Diego, CA). The experiments were performed in 2 pools of cells from two separate transfections. Cells from the first co-transfection were also plated at limiting dilution, and clones that do and do not express DN SHP2 by Western blot analysis were obtained to assess the effects of DN SHP2 on PI3K activation and complex formation.

**RESULTS**

**Thrombopoietin Activates PI3K in BaF3/Mpl Cells and Murine MKs**—Previous studies (18) in our laboratory revealed that TPO is a potent stimulus of MAPK activation. As part of that work we found that the PI3K inhibitors wortmannin and Ly294002 decreased MAPK activation significantly, suggesting that PI3K was also activated in cells exposed to TPO and that one of the downstream targets of PI3K was MAPK. To explore more formally the activation of PI3K in response to TPO, we conducted Western blot analyses to detect phosphorylation of Akt, a known downstream target of PI3K activation, and we performed direct PI3K functional assays on BaF3/Mpl cells and purified murine MKs. We found that TPO induced the activation of Akt in both cell types (Fig. 1) but not in the presence of the PI3K inhibitor Ly294002, suggesting that PI3K is activated and acts on PDK2, one of the immediate activating kinases for Akt. We also found that total cellular PI3K activity was higher in TPO-stimulated BaF3/Mpl cells than in control cells. The average PI3K activity of TPO-stimulated cells was modestly increased, 120 ± 5.0% that of unstimulated cells, as determined by PhosphorImager analysis of four separate experiments and was higher than unstimulated cells in each experiment performed. However, the amount of PI3K activity present in membrane-localized signaling complexes was markedly increased by TPO (see below).

**Thrombopoietin Induces the Association of PI3K with Several Phosphotyrosine-containing Proteins in Mpl-bearing Cell Lines**—To begin to identify the molecular mechanism(s) of TPO-induced PI3K activation, BaF3/Mpl cells were starved overnight and stimulated with TPO for 10 min, and p85 PI3K immunoprecipitates were prepared and size-fractionated, blotted, and probed with an anti-phosphotyrosine (4G10) antibody. Prior to stimulation with TPO, two phosphotyrosine-containing proteins of ~100 kDa were detected (Fig. 2). In the presence of TPO, two phosphotyrosine-containing proteins of ~170, 145, 116, 110, 100, and ~70 kDa were detected in BaF3/Mpl cells, although the ~100-kDa protein was dominant.

Since the molecular mass of members of the Gab family of adapters is ~95–110 kDa, proteins known to associate with PI3K following stimulation of the T cell and B cell receptors, IL-6 and EPO, we studied the expression of Gab proteins in BaF3/Mpl cells and primary murine MKs. We found that Gab1, Gab2, and Gab3 were all expressed in BaF3/Mpl cells, but only Gab1 and Gab3 were present in MKs (Fig. 3, A and C). In addition to Gab proteins, members of the IRS family also play a scaffolding role in many cell types. For example, IRS2 has been shown to modulate PI3K activation following stimulation of erythroid cells infected with Friend spleen focus-forming virus, which engages the EPO receptor (42), and in a hematopoietic cell line expressing the EPO receptor (43). Moreover, the molecular weight of the largest phosphotyrosine-containing proteins that co-immunoprecipitated with p85 PI3K (~170 kDa) matches that reported for IRS1 and IRS2 (Fig. 3, B and E). The formation of multiprotein signaling complexes in response to hematopoietic cytokine receptor stimulation is dependent on the phosphorylation of many of the constituent proteins. Thus, we next assessed whether TPO induced tyrosine phosphorylation of several adapters and signaling mediators and whether they associated with PI3K. As shown in Fig. 4A, Gab2 is prominently tyrosine-phosphorylated in BaF3/Mpl cells stimulated with TPO. Although BaF3/Mpl cells express small amounts of Gab1 and Gab3, they were not tyrosine-phosphorylated following exposure to TPO (data not shown). In addition, a 70-kDa phosphoprotein co-immunoprecipitated with Gab2 in these cells when stimulated with TPO; reprobing of the same immunoblot revealed it to be SHP2 (Fig. 4B). The reciprocal experiment, immunoprecipitation with anti-SHP2, and probing for Gab2 confirmed the strong association of the two phosphoproteins (Fig. 4, E and F). Gab2 was also found to
be associated with p85 (Fig. 4C). Although there are several reports describing the association of JAK2, Grb2, and SHIP with SHP2 and Gab proteins, none of these proteins were detected in our immunoprecipitates of SHP2 and Gab2 (data not shown).

As noted above, we found that BaF3/Mpl cells express IRS-2 but not IRS-1 (Fig. 3, D and E). Like Gab2, phosphorylation of IRS2 was induced by TPO stimulation of BaF3/Mpl cells (Fig. 5A). In addition, IRS2 associated with p85 PI3K (Fig. 5B) upon exposure of cells to TPO but not with SHP2 (data not shown). Moreover, IRS2 was found to associate constitutively with the Mpl receptor (Fig. 5D). Similar results were found in the human megakaryocytic cell lines, UT-7/TPO, following stimulation with TPO (data not shown). Thus, TPO induces the assembly of at least two multiphosphoprotein complexes in Mpl-bearing cell lines, one composed of Mpl, IRS2, and p85 PI3K and the other containing SHP2, Gab2, and p85 PI3K. We failed to detect reproducibly an association of p85 PI3K and Mpl by immunoprecipitation analysis.

**PI3K Is Activated in TPO-induced Multiprotein Complexes**—Previous work has shown that binding of the SH2 domain of p85 PI3K with a phosphotyrosine-containing scaffolding protein can activate PI3K. Two non-mutually exclusive mechanisms for this event have been proposed; one hypothesizes a Gab/IRS-induced conformational change in p85 allowing its activation of the p110 catalytic subunit, the other posits that the recruitment of PI3K to the membrane by the pleckstrin homology domain containing scaffolding proteins brings it in proximity of activated Ras, activating the p110 catalytic subunit, and in contact with its lipid substrates. Consistent with the involvement of this latter mechanism, TPO has been shown to activate Shc (11, 13), an adapter protein linked to Ras activation, suggesting that the multiphosphoprotein complex formed upon stimulation of BaF3/Mpl cells with TPO is an important mechanism of PI3K activation. To test whether the complexes that form in TPO-stimulated BaF3/Mpl cells were an important mechanism of PI3K activation, the capacity to phosphorylate phosphoinositol was measured in the multiprotein complexes formed in TPO-treated BaF3/Mpl cells. BaF3/Mpl cells were stimulated with the hormone for 10 min and lysed, and Gab2 or IRS2 immunoprecipitates were subjected to a direct functional kinase assay based on \[\gamma^{32}\text{P}]ATP incorporation into phosphoinositol. In contrast to the modest ~20% increase in total cellular PI3K activity noted above, TPO greatly increased PI3K activity associated with both Gab2 and IRS2.
Mechanism of TPO-induced PI3K Activation

TPO-stimulated BaF3/Mpl cells. Cell lysates were prepared from control (−) and TPO-stimulated (+) BaF3/Mpl cells (as described in Fig. 1) and immunoprecipitated with an antibody to Gab2 and sequentially probed for PY-containing proteins (A), SHP2 (B), p85 PI3K (C), or Gab2 (D). A second Western blot was prepared with SHP2-immunoprecipitated (IP) proteins and probed for PY (E), Gab2 (F), and SHP2 (G). Similar results were seen in two additional experiments. The molecular mass of standard protein markers in kDa is shown to the left of the blots.

IRS2 (Fig. 6). These results indicate that both Gab2 and IRS2 can activate PI3K by recruiting PI3K into their complexes in TPO-stimulated BaF3/Mpl cells.

SHP2 in the Complex Is Important for PI3K Activation—To investigate the physiological significance of SHP2-based complex formation in BaF3 cells, a dominant negative SHP2 (DN SHP2) construct was used to prevent SHP2-Gab2 association. The construct comprises the N-terminal SH2 domains of SHP2 that theoretically can compete with the endogenous wild type SHP2 to form the SHP2-Gab2-p85 complex. This construct was shown to inhibit SHP2/IRS1 association in Rat 1 fibroblasts (44). BaF3/Mpl cells were co-transfected with DN SHP2 and the pMX-puro plasmid as a selectable marker. Transfectants (44). BaF3/Mpl cells were co-transfected with DN SHP2 and the pMX-puro plasmid as a selectable marker. Transfectants were then selected to obtain stable cloned cell lines. Both non-expressing (clone 1) and the highly expressing clones (clone 2) were chosen for further studies (Fig. 7). TPO-induced Akt phosphorylation was reduced in the DN SHP2-expressing (clone 1) and the highly expressing clones (clone 2) were then selected to obtain stable cloned cell lines. Both non-expressing (clone 1) and the highly expressing clones (clone 2) were chosen for further studies (Fig. 7).

Since PI3K was associated with SHP2 and pp100 after stimulation with TPO (Fig. 8A), TPO-induced tyrosine phosphorylation of SHP2 and its association with p85 PI3K in MKs (Fig. 8C). To identify whether known Gab proteins were involved in TPO signaling in MKs, the isoforms of Gab present in MKs (Gab1 and Gab3) were immunoprecipitated from TPO-stimulated MK lysates and probed for phosphotyrosine-containing proteins. We failed to find P-Gab1 or P-Gab3 in MKs (Fig. 8, D and F), despite probing lysates equivalent to all the cultured MKs from up to five mice for each experimental condition (Fig. 8) and finding clear phosphorylation of Gab1 in M07e cells stimulated with stem cell factor or granulocyte-macrophage colony-stimulating factor (data not shown). However, when a p85 PI3K immunoprecipitate from TPO-stimulated MKs was probed for phosphotyrosine-containing proteins, a 100-kDa TPO-induced phosphoprotein (pp100) was detected (Fig. 8H). It is unlikely that pp100 represents one of the known IRS proteins, as the molecular mass of IRS-1, IRS-2, and IRS-4 are greater than 160 kDa and that of IRS3 is 60 kDa. In contrast, its electrophoretic mobility suggests that pp100 may be related to the Gab family of adapters.

Since PI3K was associated with SHP2 and pp100 after stimulation with TPO in primary MKs, we studied whether the formation of this complex enhances PI3K kinase activity. Since pp100 is an unknown protein, no antibody is available for direct immunoprecipitation analysis. As pp100 is the main tyrosine-phosphorylated protein associated with p85 PI3K, we immunoprecipitated total tyrosine-phosphorylated protein with the 4G10 antibody and assayed for PI3K activity with the [γ-32P]ATP PI incorporation assay. After stimulation with TPO for 10 min, PI3K activity was increased 2.1-fold in the phosphotyrosine-containing protein immunoprecipitates following TPO stimulation of MKs compared with unstimulated cell lysates (Fig. 9), suggesting that following TPO stimulation PI3K was recruited to a pp100-containing complex leading to activa-
Mechanism of TPO-induced PI3K Activation

FIG. 6. TPO induces Gab2-SHP2-p85 and IRS2-p85 complexes to activate PI3K in BaF3/Mpl cells. Gab2 or IRS2 immunoprecipitates (IP) were prepared from BaF3/Mpl cells in control (−) or TPO medium (+) and then used in a [γ-32P]ATP phosphoinositol incorporation assay. An autoradiogram of the thin layer chromatography plate is shown, with the location of the origin and 32P-phosphatidylinositol phosphate (PIP) indicated. This experiment has been performed twice with similar results.

DISCUSSION

The new findings in this paper are that TPO binding to Mpl triggers the following: 1) the association of p85 PI3K with two different adapter complexes in BaF3/Mpl cells, one composed of p85, the SHP2 phosphatase, and Gab2, and the other composed of p85 and IRS2. 2) SHP2 in the complex is important for PI3K activation. 3) A related signaling complex forms in primary MKs, also involving SHP2 and p85, the SHP2 phosphatase, and Gab2, and the other composed of p85 and IRS2. 4) Each of these complexes enhances the enzymatic activity of the associated PI3K lending physiologic relevance to the findings. 5) This pattern of PI3K activation is distinct from that seen in response to EPO, suggesting that at least some of the signaling events downstream of EPO and TPO may differ. We have also shown that Gab3 is present in both BaF3 cells and MKs but is not involved in TPO signaling. Although previous work has shown that TPO can activate PI3K in cell lines expressing the Mpl receptor, the present results extend this finding to primary cells and provide a molecular basis for the observation.

When engaged by ligand, growth factor receptors typically activate numerous signaling pathways; the aggregate of signals generated from activation of these pathways is critical for determining overall cellular response. In previously reported work, we and others have found that TPO stimulation of Mpl receptor bearing cell lines and platelets leads to activation of PI3K. In the present work we extend these findings to mature MKs. Several studies have also revealed a similar activation of PI3K in erythroid cell lines and primary cells in response to EPO, in which a direct association of the regulatory subunit of the enzyme and the EPO receptor were readily demonstrated.
nearly equivalent intensities of $^{32}$P incorporation into PI by Gab2 and IRS2 immunoprecipitates derived from identical numbers of cells, suggesting both complexes are important for TPO-induced PI3K activation in these cells.

In contrast to our results in BaF3/Mpl cells, the composition of the PI3K signaling complex responding to TPO in primary MKs differs somewhat, apparently utilizing a distinct 100-kDa scaffolding protein (Fig. 8H) along with SHP2. These results point out the importance of performing studies in primary cells, rather than relying entirely on conclusions derived from transformed cell lines. The relevance of the complex was tested in a functional PI3K assay; both Tyr(P) and P-SHP2 immunoprecipitates derived from TPO-stimulated MKs displayed enhanced activity in this assay (Fig. 9). Moreover, taken together, our results raise the issue of what determines the specificity of different hematopoietic cytokine receptors for the ever increasing number of adapter proteins. For example, several cytokines were previously shown to employ Gab1 to propagate cytokine signaling, including IL-3, the T and B cell receptors, gp130-linked cytokines, and epidermal growth factor (36, 48). However, with the cloning of Gab2, many of these same cytokines were found to also utilize Gab2, suggesting there was little specificity in adapter protein utilization. In contrast, although insulin and insulin-like growth factors recruit both IRS1 and IRS2 into signaling complexes, leptin pretreatment of cells enhances IRS1 association with PI3K but inhibits complex formation of IRS2 and PI3K (49), suggesting some specificity in adapter utilization. Here, we provide further evidence that different cytokines can employ different adapters. The EPO receptor utilizes Gab1 and Gab2 in the HCD57 cell line and Gab1 in primary erythroid progenitors (37). We found that Gab2 and IRS2 are tyrosine-phosphorylated and associate with p85 PI3K in response to TPO in BaF3/Mpl cells, but despite the presence of Gab1 in MKs, an immunologically distinct 100-kDa protein is phosphorylated and associates with p85 in these primary cells. Thus, the EPO and TPO receptors appear to utilize distinct adapter proteins in their respective primary cells, despite the common use of JAK2 by both receptors for signal initiation. These findings suggest that another factor, in addition to JAK, is probably required for specific patterns of Gab/IRS protein phosphorylation. Clearly, additional work will be required to determine the nature of these modifying signals and the identity of the $-100$-kDa phosphoprotein that associates with SHP2 and p85 in primary MKs.

Although we have shown that the TPO and EPO receptors employ different adapters in primary cells, the importance of the finding is dependent on whether or not each Gab/IRS protein gives rise to a unique set of signals. If so, then the differential use of adapters could provide a mechanism for specificity in receptor signaling. IRS proteins contain a phosphotyrosine binding domain and a larger number of putative phosphotyrosine motifs, which are different from Gabs. The recruitment of SHP2 stands as an excellent example of the potential for differential signaling; the phosphatase has been shown to associate with Gab2, a finding confirmed for the Mpl receptor in our study (Fig. 4, B and F), but does not interact with IRS-2 in our studies or in those of others (43). In addition, a recent report (50) demonstrated that IRS3 assumes a different subcellular localization than IRS1 and IRS2, which could affect its function. Furthermore, although these latter two scaffolding proteins are highly homologous and share subcellular localization patterns, the consequences of genetic elimination of IRS1...
differ from that of IRS2 (51, 52). Thus, utilizing dissimilar subsets of Gab/IRS proteins and the secondary mediators they recruit may provide a means to generate a specific set of signals from seemingly related cytokine receptors. The reason why the cellular effects of EPO stimulation differ from that induced by TPO in cells that bear receptors for both hormones (e.g., the combined erythroid-megakaryocytic colony-forming cell) may relate to adapter protein signaling differences.

Another finding reported herein is that TPO induces the activation of SHP2 in BaF3/Mpl cells, confirming the results of Saris and colleagues (53) working in 32D cells and extending the observation to primary MKs. SHP2 is a ubiquitously protein tyrosine phosphatase that is a positive regulator of growth factor signaling (45). Furthermore, it has been found to suppress the growth-inhibitory signals derived from activation of the interferon receptor (54). In this study, we have found that SHP2 is phosphorylated in response to TPO and participates in a complex with Gab/IRS-related proteins and PI3K. Since phosphorylated SHP2 can bind to Grb2 (55), our demonstration of this dominant negative form of SHP2 inhibiting the capacity of TPO stimulation. Nevertheless, by testing the PI3K activity both BaF3/Mpl cells and MKs is increased only very modestly (26). More recently, activation of PI3K was also found to be related to adapter protein signaling differences.

Mechanism of TPO-induced PI3K Activation

By bringing together both enzyme and substrate, Mpl and its TPO-induced recruitment and phosphorylation could potentially couple the Mpl receptor to activation of the SOS/Ras/Raf/MEK/MAPK pathway in an Shc-independent fashion, helping to explain previous findings whereby truncated Mpl receptors that fail to phosphorylate Shc nevertheless activated MAPK (24, 25).

To prove the physiologic relevance of the complex, a series of experiments to disrupt complex formation was performed. By using a DN SHP2 construct, we have shown that SHP2 is important for PI3K activation as reflected by Akt phosphorylation. Previous studies of insulin signaling have shown that this dominant negative form of SHP2 inhibits the capacity of insulin to activate PI3K in fibroblasts (44). These data also suggest the physiologic role of the complex in bringing SHP2 into close proximity with p85 to modulate the PI3K functions. TPO-induced SHP2/p85 association was also found in primary MKs suggesting that a similar mechanism may operate in MKs as well.

A recent report revealed a novel tyrosine-phosphorylated protein, p90, that may work downstream of SHP2 (32). However, we could not detect p90 in our complex. There-
44. Ugi, S., Maegawa, H., Kashigawi, A., Adachi, A., Olefsky J. M., and Kikkawa, R. (1996) J. Biol. Chem. 271, 12595–12602
45. Pazdruk, K., Adachi, T., and Alam, R. (1997) J. Exp. Med. 186, 561–568
46. Oh, E. S., Gu, H., Saxton, T. M., Timms, J. F., Hausdorff, S., Frevert, E. U., Kahn, B. B., Pawson, T., Neel, B. G., and Thomas, S. M. (1999) Mol. Cell. Biol. 19, 3205–3215
47. Pluskey, S., Wandless, T. J., Walsh, C. T., and Shoelson, S. E. (1995) J. Biol. Chem. 270, 2897–2900
48. Rodrigues, G. A., Falasca, M., Zhang, Z., Ong, S. H., and Schlessinger, J. (2000) Mol. Cell. Biol. 20, 1448–1459
49. Szanto, I., and Kahn, C. R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2355–2360
50. Anai, M., Ono, H., Funaki, M., Fukushima, Y., Inukai, K., Oghara, T., Sakoda, H., Onishi, Y., Yazaki, Y., Kikuchi, M., Oka, Y., and Asano, T. (1995) J. Biol. Chem. 273, 29686–29692
51. Withers, D. J., Gutierrez, J. S., Towery, H., Burks, D. J., Ren, J. M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G. I., Bonner-Weir, S., and White, M. F. (1998) Nature 391, 900–904
52. Bruning, J. C., Winnay, J., Cheatham, B., and Kahn, C. R. (1997) Mol. Cell. Biol. 17, 1513–1521
53. Mu, S. X., Xia, M., Elliot, G., Bogenberger, J., Swift, S., Bennett, L., Lappinga, D. L., Hecht, R., Lee, R., and Sarris, C. J. M. (1995) Blood 86, 4532–4543
54. You, M., Yu, D. H., and Feng, G. S. (1999) Mol. Cell. Biol. 19, 2416–2424
55. Li, W., Nishimura, R., Kashishian, A., Batzer, A. G., Kim, W. J., and Cooper, J. A. (1994) Mol. Cell. Biol. 14, 509–517
56. Shi, Z. Q., Yu, D. H., Park, M., Marshall, M., and Feng, G. S. (2000) Mol. Cell. Biol. 20, 1526–1536
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Yoshitaka Miyakawa, Ponlapat Rojnuckarin, Tania Habib and Kenneth Kaushansky

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