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Control of Thrombopoietin-Induced Megakaryocytic Differentiation by the Mitogen-Activated Protein Kinase Pathway

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Thrombopoietin (TPO) is the major regulator of both growth and differentiation of megakaryocytes. We previously showed that both functions can be generated by TPO in the megakaryoblastic cell line UT7, in which murine Mpl was introduced, and are independently controlled by distinct regions of the cytoplasmic domain of Mpl. Particularly, residues 71 to 94 of this domain (deleted in the mutant mplΔ3) were found to be required for megakaryocytic maturation but dispensable for proliferation. We show here that TPO-induced differentiation in UT7 cells is tightly dependent on a strong, long-lasting activation of the mitogen-activated protein kinase (MAPK) pathway. Indeed, (i) in UT7-mpl cells, TPO induced a strong activation of extracellular signal-regulated kinases (ERK) which was persistent until at least 4 days in TPO-containing medium; (ii) a specific MAPK kinase (MEK) inhibitor inhibited TPO-induced megakaryocytic gene expression; (iii) the Mpl mutant mplΔ3, which displayed no maturation activity, transduced only a weak and transient ERK activation in UT7 cells; and (iv) TPO-induced megakaryocytic differentiation in UT7-mplΔ3 cells was partially restored by expression of a constitutively activated mutant of MEK. The capacity of TPO to trigger a strong and prolonged MAPK signal depended on the cell in which Mpl was introduced. In BAF3-mpl cells, TPO triggered a weak and transient ERK activation, similar to that induced in UT7-mplΔ3 cells. In these cells, no difference in MAPK activation was found between normal Mpl and mplΔ3. Thus, depending on the cellular context, several distinct regions of the cytoplasmic domain of Mpl and signaling pathways may contribute to generate quantitative variations in MAPK activation.

Mpl is a member of the cytokine receptor superfamily (50, 51, 56, 57), which includes receptors for most hematopoietic growth factors, such as erythropoietin, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and most interleukins (ILs). Mpl was first isolated as an oncogenic truncated form transduced by the myeloproliferative leukemia virus (51). Its expression seems to be limited to primitive hematopoietic stem cells, megakaryocytes, and platelets (11). Thrombopoietin (TPO) was recently identified as the Mpl ligand (2, 12, 33) and was demonstrated to be the major regulator of both proliferation and differentiation of megakaryocyte progenitors in vivo and in vitro (27). In addition to this function, TPO has recently been shown to support the proliferation of primitive hematopoietic progenitor cells (31).

In a preceding study (44), we have shown that TPO can promote both proliferation and differentiation signals in the megakaryoblastic cell line UT7 (30), in which we introduced Mpl, and that these functions depend on distinct and independent regions of Mpl cytoplasmic domain (44). Emerging studies suggest that this scheme applies to a number of receptors of the cytokine superfamily, such as receptors for G-CSF (19), IL-4 (47), IL-7 (7), and the gp130 signaling chain of the IL-6 receptor (61). Upon binding to Mpl, TPO triggers a set of biochemical events common to all members of the cytokine receptor family (25), including phosphorylation on tyrosine residues of the receptor and activation of a number of transducing molecules such as members of the Janus kinase family (JAK), signal transducer and activator of transcription (STAT) factors, phosphatidylinositol 3 kinase, Shc, and tyrosine phosphatases (1, 14, 15, 21, 23, 37, 38, 41).

Activation of closely related serine-threonine mitogen-activated protein kinases (MAPK) has been shown to be a key event in signal transduction by growth factors. The MAPK family comprises the extracellular signal-regulated kinases (ERKs) $p44^{mapk}$ (ERK1) and $p42^{mapk}$ (ERK2) and stress-activated protein kinases p38 and Jun kinases (reviewed in reference 58). MAPKs are activated by dual phosphorylation at neighboring threonine and tyrosine residues, and dephosphorylation of either residue results in MAPK inactivation. The general scheme of ERK activation involves a cascade of phosphorylation events initiated by stimulation of the *ras* proto-oncogene (34, 49). The main postreceptor pathway leading to Ras protein activation involves the adaptor proteins Grb2 and Shc, which allow the transport of the GTP-GDP exchange factor SOS1 from the cytoplasm to the membrane (34, 49). Ras in turn activates members of the Raf family by a not yet fully understood mechanism, which may implicate interaction with the activated GTP-bound form of Ras and oligomerization. Raf then phosphorylates downstream kinases called MAPK-activating enzymes (MAPKK, or MEK) at serine or threonine residues, which in turn catalyze dual phosphorylation and activation of ERK. Activated ERKs translocate to the nucleus (53), where they phosphorylate transcription factors capable of mediating immediate-early gene induction (reviewed in reference 55).

Recently, the importance of the MAPK pathway in mediating specific signaling functions of tyrosine kinase receptors has been approached by overexpressing dominant negative or constitutively activated mutants of MEK. These studies clearly established the requirement for the MAPK pathway in fibro-

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blast proliferation and transformation (4, 8, 35, 39). The MAPK pathway is also necessary for neurite outgrowth of PC12 pheochromocytoma cells (8, 46, 53), and the duration and amplitude of the MAPK signal triggered downstream of tyrosine kinase receptors have been shown to be critical for determining whether these cells proliferate or differentiate (8, 46, 53, 54).

In hematopoietic cells, the role of the MAPK pathway is not clear. Oncogenic Ras increases the self-renewing capacity of some progenitor cells without blocking their capacity to undergo terminal differentiation (22, 43), and it could do so by acting on cascades different from MAPK. More recently, the MAPK pathway has been clearly demonstrated to control the differentiation of immature thymocytes downstream of the pre-T-cell receptor (9). As far as cytokine receptors are concerned, ERK activation has been reported for most members, including Mpl (25, 38, 60), but has been shown to be either necessary or dispensable for full proliferation (25). To our knowledge, direct evidence that the MAPK pathway is responsible for maturation signals induced by hematopoietic growth factors is lacking. In the present study, we addressed the role of the MAPK pathway in TPO-mediated functions by expressing the murine full-length Mpl receptor or Mpl mutants in two different factor-dependent cell lines which respond to TPO stimulation either by proliferating (BAF3 cells) (1, 14, 21) or by differentiating towards the megakaryocyte lineage (UT7 cells) (44). We demonstrate that the MAPK pathway is required for TPO-induced maturation signals but is dispensable for proliferation. Furthermore, we show that the capacity of TPO to induce megakaryocytic differentiation is dependent on the intensity and duration of the MAPK signal and that, depending on the cell type in which Mpl was expressed, several distinct regions of the intracellular domain of Mpl may contribute to this signal.

MATERIALS AND METHODS

Antibodies and reagents. Rabbit polyclonal antibodies anti-ERK1 ($p44^{mapk}$) and anti-ERK2 ($p42^{mapk}$) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.); anti-Shc and anti-MEK were from Transduction Labs (Lexington, Ky.). Antiphosphotyrosine monoclonal antibody 4G10 was a gift of B. Drucker (Portland, Oreg.). Monoclonal antibody M1, specific for the flag epitope tag sequence, was kindly donated by D. Cosman (Immunex, Seattle, Wash.), and 12CA5, specific for the influenza virus hemagglutinin (HA) tag, was obtained from BAbCO (Richmond, Calif.). For immunofluorescence analysis, the following antibodies were purchased from Immunotech (Marseille, France): anti-CD41 (gpIIb), anti-CD61 (gpIIIa), and anti-CD42b (gpIb). Fluorescein isothiocyanate conjugated goat $F(ab')_2$ fragments specific for mouse immunoglobulin G and purified goat immunoglobulin G were obtained from Sigma (St. Louis, Mo.).

The specific MEK inhibitor PD98059 (16) was purchased from New England Biolabs Inc. (Beverly, Mass.). Phorbol myristate acetate (PMA) was obtained from Sigma. Stock solutions were prepared at 1 mg/ml for PMA and 10 mM for PD98059 in dimethyl sulfoxide (DMSO).

Cell culture and cytokines. The murine cell line BAF3 (40) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 4% conditioned medium from WEHI-3B cells as a source of murine IL-3. The human megakaryoblastic cell line UT7 (30) was cultured in α-minimum essential medium supplemented with 10% FCS and 2.5 ng of recombinant human GM-CSF per ml. Murine TPO was obtained as a serum-free medium from baby hamster kidney cells engineered to stably express murine TPO (33) and was provided by Don Foster (Zymogenetics, Inc., Seattle, Wash.). Before cytokine stimulation, BAF3 or UT7 cells expressing the various Mpl constructs were deprived of growth factors by being washed three times in serum-free medium and maintained in Iscove's medium supplemented with 0.4% bovine serum albumin and 20 μg of transferrin per ml in the absence of cytokine for 18 h at 37°C.

Plasmid constructions. The cDNA encoding the full-length murine c-Mpl receptor (mplWt) with a flag epitope tag sequence at the N-terminus and the truncation mutant mplΔ3 lacking residues 576 to 599 in the intracellular domain of Mpl, were obtained as described previously (44). The cDNAs for mplWt and mutant mplΔ3 were subcloned into the *Sal*I site of the retrovirus expression vector pBabepuro carrying the puromycin resistance gene.

The expression vector for the constitutively active MAPKK 1 (MEK1) mutant, in which serines 218 and 222 were both replaced with glutamic acid (MEK1-SS/DD) in frame with the HA epitope (4, 39), was kindly donated by A. Brunet and J. Pouyssegur (CRNS-UMR 134, Nice, France). MEK1-SS/DD was subcloned in the pcDNAneo vector (Invitrogen) carrying the neomycin resistance gene.

Cell transfections. A total of 5×10^6 cells (BAF3 or UT7) were washed three times in serum-free medium and resuspended in 800 μl of 10 mM phosphate buffer (pH 7.6), containing 120 mM KCl, 0.15 mM $CaCl_2$, 25 mM HEPES, 2 mM EGTA, 5 mM $MgCl_2$, 5 mM glutathione, and 2 mM ATP and mixed with 10 μg of plasmid DNA encoding mplWt or mutants. After 10 min at room temperature, cells were electroporated at 250 V and 960 μF with a Gene Pulser (Bio-Rad, Hercules, Calif.), and incubation was continued for 10 min at 37°C. Cells were then seeded in culture flasks containing the appropriate medium and cytokine (WEHI-conditioned medium for BAF3 cells and GM-CSF for UT7 cells). After 24 h, transfected cells were selected in medium containing 1 μg of puromycin (Sigma) per ml and seeded at various dilutions in 24-well plates or kept in flasks to obtain polyclonal cell populations. After about 2 weeks, cells were expanded and analyzed for Mpl expression by flow cytometry after being stained with antiflag antibody. Positive clones were kept for further studies. In some instances, when Mpl expression was too low, the cells were subjected to one round of fluorescence-activated cell sorting with antiflag antibody on an ELITE flow cytometer (Coultronics).

Constitutively active MEK1-SS/DD mutants in the pcDNAneo vector or the pcDNAneo vector alone were introduced into UT7-mplΔ3 cells as described above, but cells were selected in 1 mg of G418 (Gibco BRL, Gaithersburg, Md.) per ml instead of puromycin. Expression of the construct was assessed by Western blotting with anti-HA antibodies as described below, and positive clones were analyzed for MEK activity and their capacity to differentiate in the presence of TPO.

Proliferation and differentiation assays. Cells expressing Mpl constructs were washed three times to remove growth factors, resuspended at 5×10^4/ml, and plated in six-well plates in medium containing 10% FCS and either 2.5 ng of GM-CSF per ml or 400 U of TPO per ml in the presence or absence of various concentrations of MEK inhibitor diluted in DMSO or in DMSO alone. At the indicated time intervals, cells were counted, and viability was determined by trypan blue exclusion. Cultures were split twice a week to maintain cell concentrations below 4×10^5/ml. Megakaryocytic differentiation was assessed after 7 days of culture in TPO by measuring the expression of the megakaryocyte-specific markers CD41, CD61, and CD42b on the cell surface by flow cytometry, as described previously (44).

Immunoprecipitation and Western blotting. Deprived BAF3-mpl and UT7-mpl cells were stimulated with either TPO (10 to 1,000 u/ml), PMA (100 ng/ml), or medium alone for various times at 37°C and lysed in lysis buffer containing 20 mM Tris (pH 7.5), 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM Na orthovanadate, 2 mM sodium pyrophosphate, 1 mM PMSF, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, and 1 μg of pepstatin per ml. Lysates were centrifuged at 17,000 × *g* for 15 min at 4°C. For immunoprecipitation, clear cell lysates were incubated for 2 h at 4°C with the desired antibody, and immune complexes were collected by incubation with protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) for 1 h at 4°C. Immunoprecipitates were washed four times in lysis buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). MAPK detection was performed directly with whole-cell extracts by loading lysates from 2×10^5 cells on SDS–12% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies to either ERK1, Shc, HA, MEK, or phosphotyrosine. Detection was performed with secondary antibodies coupled to horseradish peroxidase and an enhanced chemiluminescence detection system (Amersham).

Protein kinase assays. Lysates from BAF3-mpl or UT7-mpl cells stimulated with TPO or PMA were immunoprecipitated with either a mixture of anti-ERK1 and -ERK2 or anti-MEK antibodies as described above. Immune complexes were washed twice in lysis buffer and then twice in kinase buffer: 25 mM HEPES (pH 7.5) containing 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 2 mM dithiothreitol, and 0.1 mM sodium orthovanadate. Immune complex kinase assays were performed at 30°C for 20 min by incubating immunoprecipitates in a total volume of 50 μl of kinase buffer containing 50 μM ATP and 3 μCi of [γ-^{32}P]ATP and 2 to 5 μg of either myelin basic protein (MBP) or glutathione *S*-transferase (GST)-[K17A]ERK1 fusion protein (Upstate Biotechnology) as substrates for measurement of ERK and MEK activities, respectively. The reactions were terminated by boiling the samples in Laemmli sample buffer, and the products were resolved by SDS-PAGE on 15% polyacrylamide gels. Radioactive bands were visualized by autoradiography and quantitated by phosphorimaging.

RESULTS

TPO induces a strong and sustained activation of MAPK in UT7 cells. To assess the role of the MAPK pathway in Mpl signaling, we analyzed the activation of the p42 and p44 MAPKs (ERK1 and ERK2) in UT7 cells in which we have introduced exogenous wild-type murine Mpl (UT7-mplWt [Fig. 1]). UT7 is a megakaryoblastic cell line of human origin, dependent on GM-CSF or EPO for its growth (30). We de-

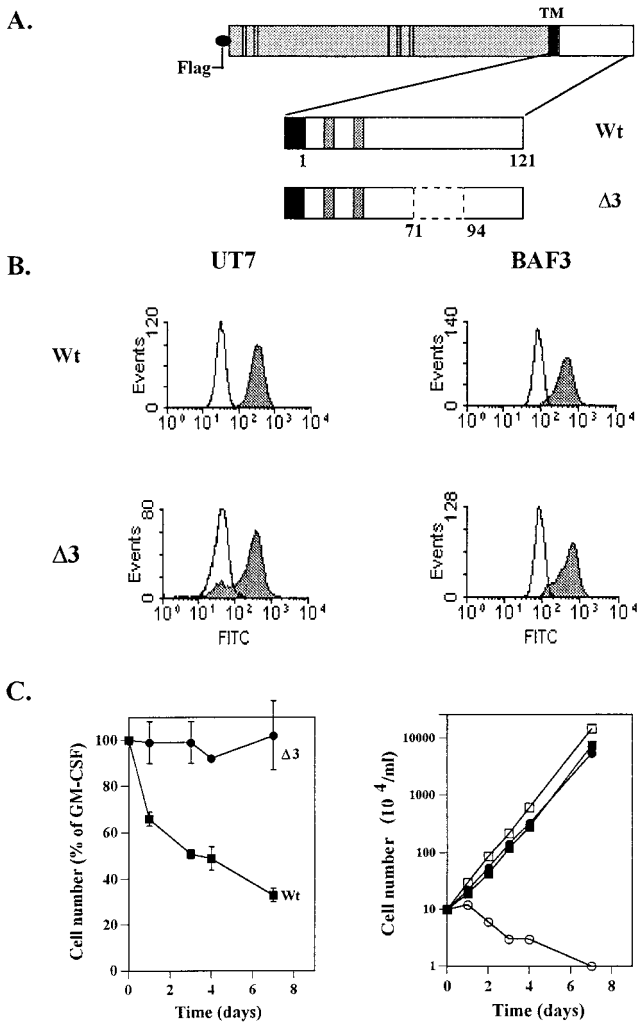

FIG. 1. Mpl receptor mutants. (A) Schematic representation of full-length (Wt) and mutant Mpl (Δ3). Numeration starts at the first amino acid of the intracellular domain. TM, transmembrane domain. The conserved Box1 and Box2 motifs of the cytoplasmic domain are represented by shaded grey areas. (B) Cell surface expression of the various Mpl constructs in UT7 and BAF3 cells analyzed by flow cytometry. Cells cultured in GM-CSF (UT7 cells) or IL-3-containing medium (BAF3 cells) were stained either with antiflag antibody M1 (shaded peaks) or control antibody (open peaks). (C) Long-term growth of UT7 (left panel) and BAF (right panel) cells expressing mplWt (■) or mplΔ3 (●) forms. Cells were shifted to medium containing 400 U of TPO per ml, and the number of viable cells was determined at the indicated times. For UT7 cells, results are expressed as a percentage of the cell number obtained after 7 days of culture in the presence of GM-CSF. For BAF3 cells, absolute numbers of viable cells are indicated. ○, parental BAF cells cultured in the presence of TPO. The growth response in IL-3-containing medium is shown for BAF3-mplWt cells (□) and was similar for all transfectants or parental cells.

scribed in a previous study that UT7 cells expressing mplWt can be induced to differentiate towards a more mature megakaryocytic phenotype upon culture in the presence of TPO (44). Maturation was characterized by changes in cell morphology and a three- to fivefold increase in megakaryocyte-specific differentiation antigens CD41 (gpIIb), CD61 (gpIIIa), and CD42b (gpIb). At the same time, growth was strikingly reduced (44) (Fig. 1C).

ERK activation was analyzed by detecting the mobility shift resulting from hyperphosphorylation in an immunoblot with an anti-ERK1 antibody which recognizes both ERK1 and ERK2. Kinase activity was determined by immunoprecipitating ERK1 and ERK2 and measuring their ability to phosphorylate MBP. As shown in Fig. 2, TPO induced a strong and sustained activation of both ERK1 and ERK2 MAPK in UT7-mplWt cells. ERK kinase activity increased by 17- to 23-fold after 60 min of treatment with TPO. Kinetic studies showed that activation was relatively slow, being detected after about 15 min at 37°C and reaching a maximum by 1 to 2 h of TPO stimulation (Fig. 2A and B). MAPK activation induced by TPO in UT7-mplWt cells was largely persistent. Indeed, no decrease in the phosphorylation or kinase activity of ERK1 and ERK2 was detected until 4 h of stimulation with TPO (Fig. 2A and B). In addition, a shift in electrophoretic mobility could still be detected in cells grown in TPO for 1 to 4 days, while under the same conditions, no activation was seen in GM-CSF-containing cultures (Fig. 2C). ERK activity triggered by TPO was similar to that induced by the phorbol ester PMA (Fig. 2B), a known powerful activator of MAPK.

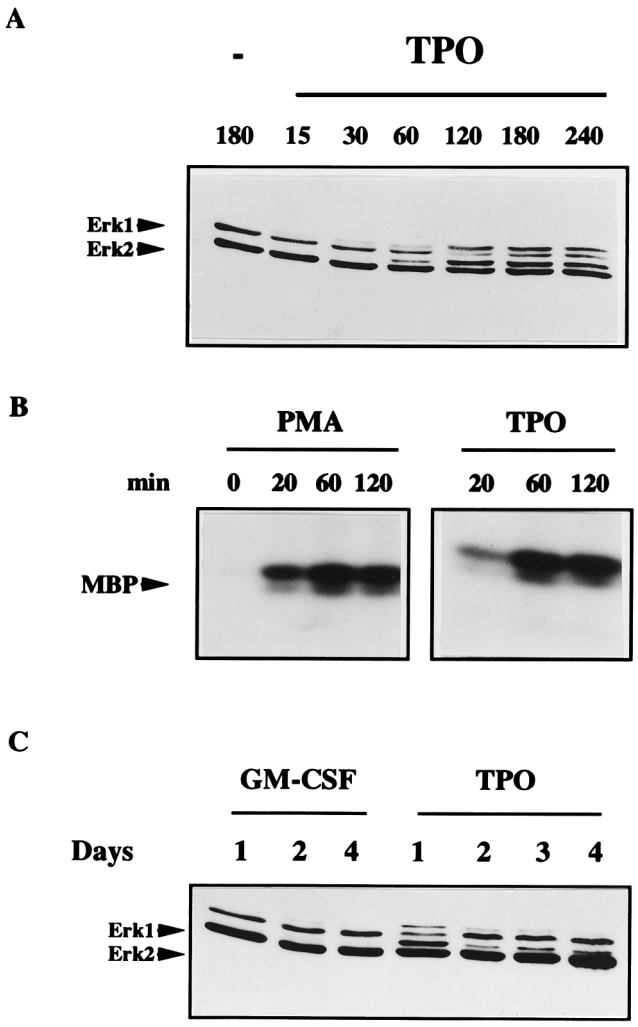

FIG. 2. MAPK activation in UT7-mplWt cells detected by electrophoretic shift by Western blotting of whole-cell lysates with anti-ERK1 antibody (A and C) or by kinase assay in anti-ERK1 plus -ERK2 immunoprecipitates (B). (A and B) UT7-mplWt cells were deprived of growth factors overnight at 37°C and stimulated with TPO (1,000 U/ml) or PMA (100 ng/ml) for the indicated times. (C) UT7-mplWt cells were washed three times and resuspended in complete medium containing either GM-CSF (2.5 ng/ml) or TPO (400 U/ml), and MAPK activity was tested at the indicated days.

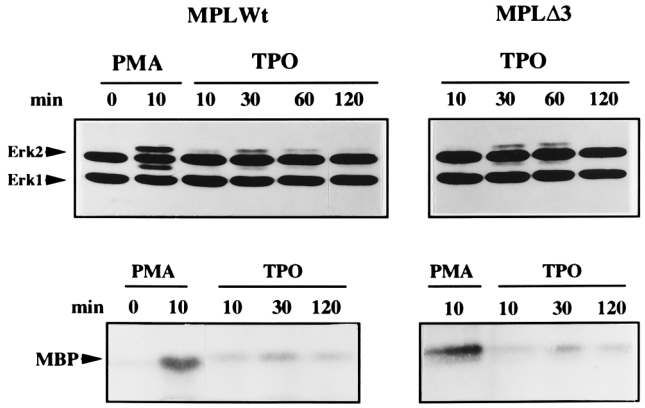

FIG. 3. MAPK activation in BAF3 cells expressing normal Mpl or mplΔ3 mutant receptors. BAF3-mplWt or BAF3-mplΔ3 cells were deprived of growth factors and stimulated with either medium alone, PMA (100 ng/ml), or TPO (1,000 U/ml). At the indicated times, ERK activity was assessed by mobility shift or kinase assays as described in the legend to Fig. 2.

TPO induced a weak and transient activation of MAPK in BAF3-mpl cells. To delineate the importance of this sustained MAPK activation induced by TPO in UT7 cells in causing growth slowing and differentiation, we introduced Mpl in another cellular context—the BAF3 cells (Fig. 1B). Consistent with previous studies (1, 14, 21), BAF3-mplWt cells but not parental cells proliferated continuously in the presence of TPO (Fig. 1C). No change in growth rate, cell morphology, or expression of CD61 antigen was observed over a period of at least a month of culture in TPO (data not shown). The patterns of MAPK activation in TPO-stimulated BAF3-mplWt and UT7-mplWt cells were very different. In BAF3-mplWt cells, TPO triggered a rapid and transient ERK activation, peaking after 30 to 60 min of stimulation and then declining gradually to reach almost basal levels by 2 h (Fig. 3). As for UT7 cells, the shift in mobility of ERK1 and ERK2 paralleled their kinase activity. In addition, by contrast with UT7 cells, even at its peak level, MAPK activation induced by TPO in BAF3-mplWt cells (3-fold increase in kinase activity) was weak compared to that induced by PMA (12-fold increase in kinase activity) (Fig. 3).

The same domain of Mpl is involved in megakaryocytic maturation and strong, long-lived activation of MAPK in UT7 cells. In a previous study (44), we developed a series of deletion mutants of the intracellular domain of Mpl and showed that residues 71 to 94 (deleted in mutant mplΔ3) were required for differentiation of UT7 cells but dispensable for proliferation. In UT7-mplΔ3 cells, no change in the surface expression of megakaryocyte-specific antigens was induced after a 7-day-treatment with TPO (44). TPO was able to sustain the long-term proliferation of both UT7-mplΔ3 and BAF3-mplΔ3 transformants, and no decrease in growth rate of UT7-mplΔ3 was observed (44) (Fig. 1C). To further examine the role of the MAPK pathway in TPO-induced signaling, we studied the capacity of mplΔ3 to activate MAPK in both UT7 and BAF3 cells. MplΔ3 was introduced in UT7 and BAF3 cells, and cell populations expressing levels of exogenous flag-Mpl equivalent to those of UT7-mplWt and BAF3-mplWt were selected (Fig. 1B).

The capacity of TPO to activate ERK1 and ERK2 was strikingly different in UT7 cells expressing mplΔ3 and the full-length Mpl. In UT7-mplΔ3 cells, TPO induced only a faint mobility shift and an increase in the kinase activity of both MAPKs (Fig. 4). At the peak response (around 60 min after TPO stimulation), kinase activity was increased by 3.5-fold (range 1.5- to 6-fold) in response to TPO. However, this response was not maintained, and almost no shift in mobility could be detected after 1 day in culture of UT7-mplΔ3 cells in TPO-containing medium (Fig. 4A and B). The weak activation induced by TPO was not due to a general defect in MAPK activation in UT7-mplΔ3 cells, since PMA was fully able to activate MAPK in these cells (Fig. 4A).

The weak and short-lived activation of MAPK induced by TPO in UT7-mplΔ3 transformants appeared similar to that observed upon TPO stimulation of BAF3-mplWt cells (Fig. 3). Furthermore, we found that mplΔ3 could transduce a MAPK activation signal in BAF3 cells that is absolutely similar to that induced by mplWt in the same cells (Fig. 3). This suggested that residues 71 to 94 of the intracellular domain of Mpl were not necessary for triggering the MAPK pathway but might control the intensity and duration of this signal in UT7 cells.

Altogether, these results indicate that TPO can maintain strong activation of the MAPK pathway specifically in UT7 cells. In these cells, several distinct domains of Mpl seem to contribute to MAPK activation, the region delimited between amino acids 71 and 94 of the cytoplasmic domain of Mpl being involved in the control of level and duration of this signal. Since the same region is also necessary for TPO-induced megakaryocytic differentiation and growth arrest in UT7 cells, these data suggest that a strong and sustained activation of this pathway would be required for the maturation function of Mpl.

Shc phosphorylation is not impaired in UT7 cells expressing the mplΔ3 mutant. Phosphorylation of the Shc adaptor protein has been shown to be an important element linking a wide variety of receptors to the Ras-MAPK signaling pathway (42, 49). To examine the possibility that Shc was involved in the control of the duration and intensity of the MAPK activation, Shc activation was assessed in UT7 cells expressing the full-length or mplΔ3 mutant receptors. Consistent with previous studies in other cell types (1, 14, 15, 21, 23, 38), we observed that Shc was rapidly phosphorylated in UT7-mplWt cells in response to TPO stimulation. Four bands phosphorylated on tyrosine with sizes of 145, 67, 57, and 50 kDa were detected by Western blotting of Shc immunoprecipitates with antiphosphotyrosine antibodies (Fig. 5A). The three low-molecular-mass species corresponded to the various forms of the Shc protein described previously (42), since they reacted with anti-Shc

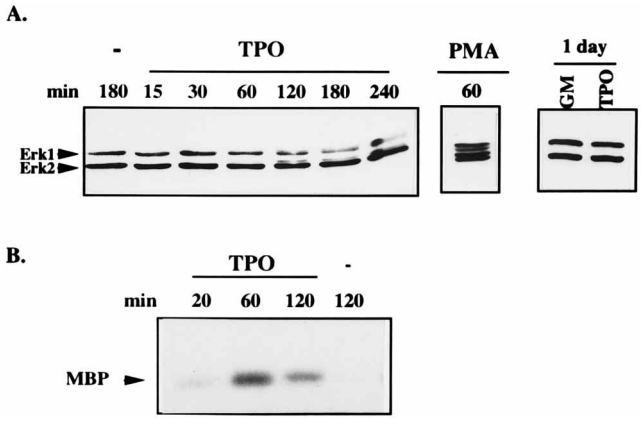

FIG. 4. TPO-dependent induction of ERK activation in UT7 cells expressing the mplΔ3 mutant. ERK activation was tested by mobility shift assay of whole lysates (A) or kinase activity of ERK immunoprecipitates (B) from cells deprived of growth factors and stimulated for the indicated times at 37°C with medium alone, TPO (1,000 U/ml), or PMA (100 ng/ml). For 1-day stimulation, cells were incubated in full medium as described in the legend to Fig. 2.

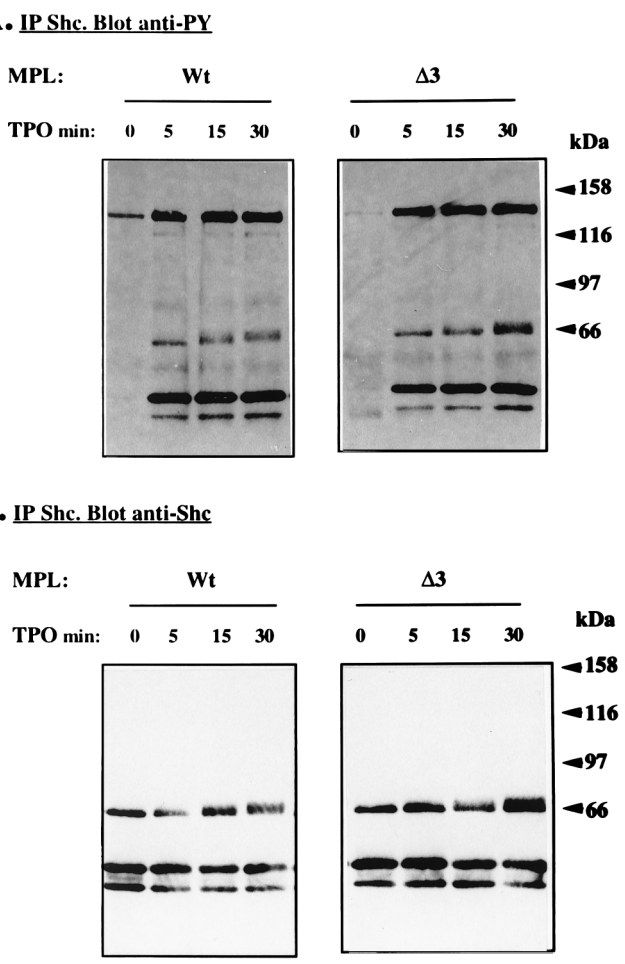

FIG. 5. Shc phosphorylation in UT7 cells expressing mplWt or mplΔ3. Cells were deprived of growth factors and stimulated with TPO (1,000 U/ml) or left unstimulated for the indicated times at 37°C. Anti-Shc immunoprecipitates (IP) were prepared and analyzed in Western blots with antiphosphotyrosine (anti-PY) antibody (A) or anti-Shc antibody (B). Standard molecular masses are indicated by arrowheads.

antibodies (Fig. 5B). In addition, a TPO-dependent shift in mobility of the 67-kDa species was observed in both antiphosphotyrosine and anti-Shc immunoblots. The 145-kDa band probably corresponds to the Shc-associated phosphoprotein SHIP identified in several cytokine receptor systems (10).

Figure 5 shows that identical patterns of phosphorylation of Shc and p145-Shc-associated protein were induced in UT7 cells expressing mplWt or mplΔ3, indicating that amino acids 71 to 94 of the intracellular domain of Mpl are not involved in Shc activation. Thus, the defect in ERK activation of the mplΔ3 in UT7 cells is independent of Shc.

Rescue of TPO-induced megakaryocytic differentiation in UT7-mplΔ3 cells by stable expression of a constitutively activated MEK mutant. To determine at which level the MAPK signaling cascade was altered in UT7-mplΔ3 transformants, we stably introduced a constitutively activated HA-tagged mutant of MEK1 in these cells. This mutant has been previously shown to display a constitutive activity towards MAPK in vitro and to induce growth factor-independent proliferation of fibroblasts (4, 39). As a control, UT7-mplΔ3 cells were transfected with empty vectors. Several neomycin-resistant clones and mass populations of cells expressing empty vector (Δ3-E) or a MEK mutant (Δ3-AMEK) were obtained. Expression of activated MEK protein varied from one clone to another, as detected by Western blotting of whole-cell lysates with anti-HA antibodies (Fig. 6A). Under basal conditions (i.e., cells grown in full medium containing GM-CSF), MEK activity could be detected in Δ3-AMEK clones but not in Δ3-E cells (Fig. 6B). Stable expression of an activated form of MEK did not affect the rate of cell growth in GM-CSF, nor did it lead to growth factor-independent proliferation of cells (data not shown). Activated MEK did not change the basal levels of surface expression of megakaryocytic antigens in cells cultured in GM-CSF. However, the capacity of TPO to induce megakaryocytic markers in UT7 cells expressing mplΔ3 was restored partially by expression of the constitutively activated MEK mutant (Fig. 6C). Interestingly, the extent of the TPO-induced increase in CD41 and CD61 varied proportionally with the amount of activated MEK protein expressed in the cells, the expression of CD41 under TPO treatment reaching 44 and 28% of the UT7-mplWt cell response in clone Δ3-AMEK-1 and clone Δ3-AMEK-2, respectively. Likewise, the reduction in cell growth observed in TPO cultures of UT7-mplΔ3-AMEK transformants paralleled the quantitative expression of activated MEK protein (Fig. 6D).

These results further confirm that a continuous activation (or absence of inhibition) of the MAPK pathway is necessary for TPO-mediated differentiation function. They also indicate that the defect in the mplΔ3 mutant leading to weak MAPK activation lies on the MEK-MAPK pathway.

The MAPK pathway is necessary for TPO induction of megakaryocytic differentiation but is dispensable for proliferation. To further confirm the importance of the MAPK cascade in TPO signaling, we tested the effect of the MEK inhibitor PD98059 on the capacity of TPO to mediate differentiation and proliferation functions in UT7 and BAF3 cells expressing Mpl. This inhibitor has been shown to inhibit in a specific manner MEK1 and MEK2 kinases with 50% inhibitory concentrations of 5 to 10 and 50 μM, respectively, while having no effect on the parallel stress-activated kinase pathway (16). Figure 7A shows that PD98059 inhibited most of the increase in megakaryocytic markers induced after 7 days of TPO stimulation of UT7-mplWt cells when added at a concentration of 10 μM in cell culture medium. The TPO-mediated increments in CD41, CD42b, and CD61 expression were inhibited by 69% ± 5% (n = 5), 91% ± 5% (n = 5), and 66% ± 5% (n = 3), respectively, compared to that in cells incubated with TPO alone. In addition, TPO-induced changes in cell morphology, such as adherence and size increase, were completely abolished in the presence of PD98059 in the cultures. No change in CD41, CD42b, or CD61 levels were observed on cells treated with GM-CSF and PD98059 compared to those in cells treated with GM-CSF only. At the concentration used for the differentiation assays, PD98059 was able to completely reverse the slow-growing effect of TPO on UT7-mplWt cells, which now grew at the same rate in TPO and in GM-CSF (Fig. 7B). This demonstrates that the inhibitory effect of PD98059 on TPO-mediated differentiation is not due to cell toxicity. Dose-response studies showed that the inhibitor was already active at inhibiting TPO-induced growth arrest of UT7-mplWt cells at a concentration of 0.1 μM (data not shown).

To further assess the role of the MAPK pathway in TPO-induced proliferation, we tested the effect of MEK inhibitor in two systems in which TPO has no growth-slowing effect, i.e., in which no cell differentiation occurred: UT7-mplΔ3 and BAF3-mplWt cells. The proliferation induced by TPO was altered in neither of these systems, even after prolonged treatment with PD98059 (Fig. 7B). PD98059 induced a slight reduction of GM-CSF-mediated growth of UT7 cells (32% ± 6% [n = 5] decrease in cell number after 7 days of culture).

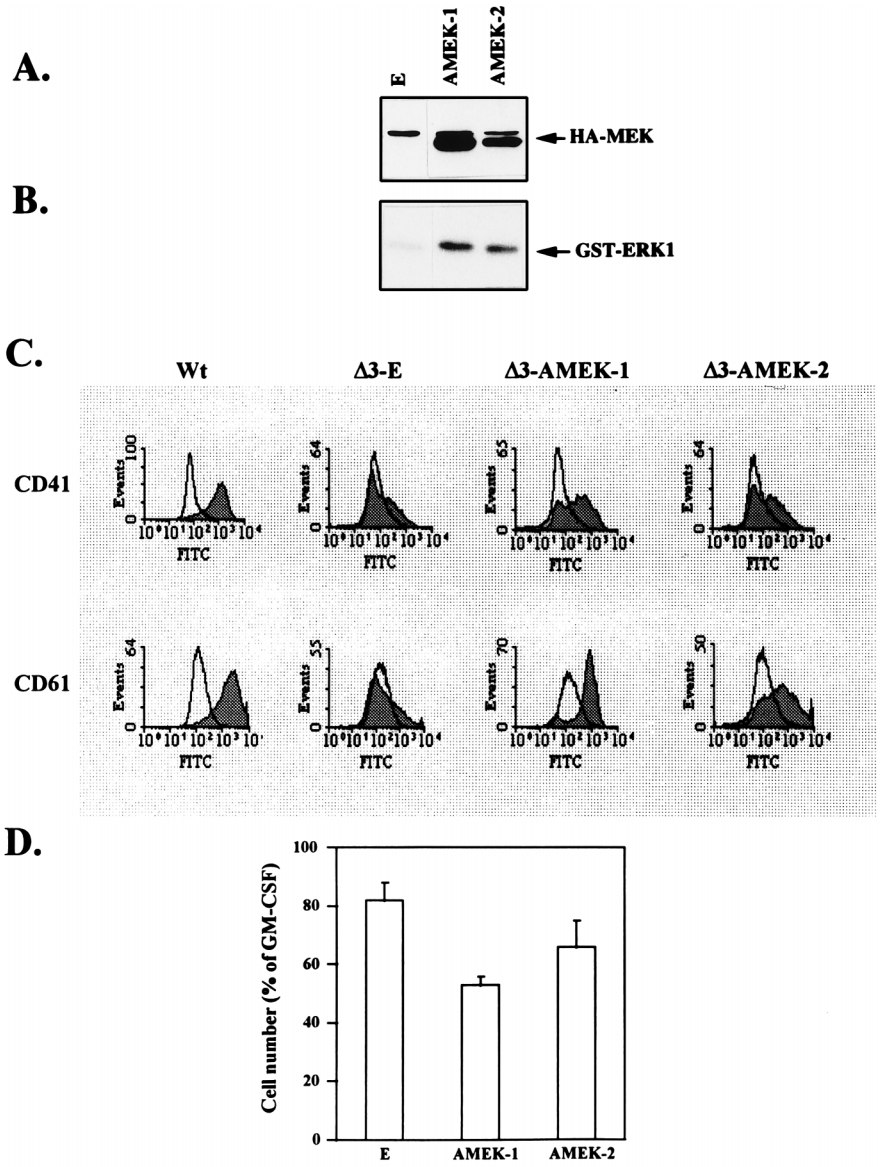

FIG. 6. Rescue of TPO-mediated differentiation of UT7-mplΔ3 cells by stable expression of the constitutively activated MEK mutant. Results from two different clones of UT7-mplΔ3 cells transfected with constitutively activated MEK (clones AMEK-1 and AMEK-2) and one clone of cells transfected with empty vector (E) are represented. (A) Expression of exogenous activated MEK detected by Western blotting with anti-HA antibody. (B) MEK kinase activity in nonstimulated cells. Lysates from cells continuously growing in GM-CSF were immunoprecipitated with anti-MEK antibody, and kinase activity was assessed by phosphorylation of kinase-dead GST-ERK1 fusion protein as described in Materials and Methods. (C) Flow cytometry analysis of expression of megakaryocytic markers CD41 and CD61 assessed after 7 days of culture of the various cells in medium containing 400 U of TPO per ml. Open peaks, GM-CSF; shaded peaks, TPO. (D) Number of viable cells determined after 7 days of culture in TPO-containing medium, expressed as a percentage of cells counted in GM-CSF cultures.

As shown in Fig. 8, a concentration of 10 μM PD98059 completely inhibited ERK1 and ERK2 electrophoretic mobility shift as well as kinase activity induced by TPO stimulation of UT7-mplWt cells at all times of incubation tested. Similar results were obtained with UT7-mplΔ3 cells. Thus, the MAPK cascade is dispensable for TPO-mediated proliferation but is required for growth reduction and differentiation of UT7-mplWt cells.

DISCUSSION

In the past few years, an attempt has been made to determine whether different growth factor receptors can activate distinct signaling pathways, and whether one of these pathways will specifically lead to cell proliferation or differentiation decisions has become a central issue. A new concept in this field has been introduced recently by several groups demonstrating that cellular responses triggered by receptors of the tyrosine kinase family could be determined by quantitative differences in the activation of a single signaling pathway (8, 46, 53, 54). We generalize this concept here by showing for the first time that such quantitative variations in MAPK activation also seem to be a critical step in signaling by a member of the cytokine receptor family: murine Mpl introduced in the megakaryoblastic cell line UT7 allows transduction of TPO-induced megakaryocytic maturation, reduction in the growth rate, and strong and sustained activation of MAPK. A specific region of Mpl was found to be involved in the capacity to sustain strong

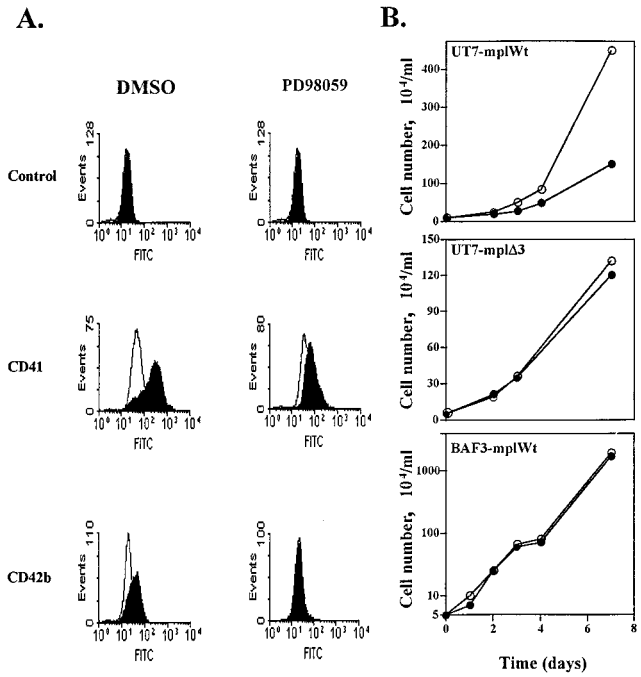

FIG. 7. Inhibition of TPO-mediated differentiation but not proliferation by the MEK inhibitor PD98059. (A) Flow cytometric analysis of CD41 and CD42b antigen expression on UT7-mplWt cells incubated for 7 days with 2.5 ng of GM-CSF per ml (open peaks) or 400 U of TPO per ml (solid peaks) in the presence of DMSO or 10 μM PD98059 diluted in DMSO. (B) Long-term growth of UT7 cells expressing mplWt or Δ3 mutants and BAF3-mplWt cells cultured in TPO in the presence of 10 μM PD98059 (open circles) or DMSO (solid circles).

MAPK activation and to trigger megakaryocytic maturation in UT7 cells. UT7 cells grown in the presence of GM-CSF or EPO display, respectively, the features of promyelocytes or erythroblasts (30). In our system, GM-CSF was not able to sustain strong MAPK activation. In addition, EPO has been shown previously to induce a transient MAPK activation in UT7 cells (20). Thus, variations in amplitude and duration of MAPK activation may be one step allowing specific transduction of megakaryocytic maturation events by TPO.

The amplitude and duration of the MAPK activation signal transduced by Mpl depended on the cellular context in which this receptor was introduced. Indeed, in contrast to UT7 cells, in BAF3-mpl cells, TPO induced a weak and transient activation of MAPK. In the case of tyrosine kinase receptors, differences in receptor numbers have been shown to markedly affect the duration of ERK activation, because receptors for epidermal growth factor become able to trigger both neuronal differentiation and long-lived ERK activation when overexpressed in PC12 cells (54). This does not seem to explain the differential responses of UT7 and BAF3 cells to TPO, since Mpl levels at the surface of BAF3 and UT7 cells were similar. Furthermore, the mplΔ3 mutant triggered only a weak activation of MAPK, similar to that in UT7 and BAF3 cells, even though it was present at high levels on both cells. Rather, different levels and kinetics of ERK activation may depend on the cellular context (i.e., the presence or absence of mediators leading to or controlling MAPK activation). Indeed, previous studies have shown that several distinct independent routes leading to ERK activation can coexist in one cell and that, depending on the cell type, growth factors may connect to one or more of these pathways (5, 6, 26, 48). For example, platelet-derived growth factor can trigger ERK activation in fibroblasts through protein kinase C (PKC)-dependent or -independent routes; in fibroblasts, but not in BAF3 cells, ERK activation is dependent on phosphatidylinositol-3-kinase binding to the receptor. Our results show that a constitutively activated mutant of the MAPKK MEK1 allowing sustained ERK activation could restore, at least partially, maturation signals induced by TPO in UT7 cells expressing the defective mplΔ3 mutant. This suggests that TPO provides sustained MAPK activation in UT7 cells through activation of an element of the MAPK pathway. This could involve various signaling effectors downstream of Mpl which would be either absent or present in too small amounts in the BAF3 cells.

One of the pathways that link cytokine receptors to the Ras-MAPK pathway involves the Shc adaptor molecule (42, 49). In agreement with previous studies from other laboratories (1, 14, 15, 21, 23, 37, 38), we found that TPO induced tyrosine phosphorylation of Shc in Mpl-expressing cells. However, Shc phosphorylation was not affected in UT7-mplΔ3, in which TPO-induced long-lasting MAPK activation was severely impaired. This suggests either that TPO triggers MAPK activation in UT7 cells in a way independent of Shc or that Shc activation in UT7 cells, although necessary, is not sufficient to maintain a strong MAPK activation. The capacity of the mplΔ3 mutant to trigger a weak activation of ERK in UT7 cells similar to that induced in BAF3 cells supports the latter hypothesis and suggests that the weak ERK activation in UT7-mplΔ3 cells would result from Shc activation. Introduction of Mpl mutants deficient for Shc activation or dominant negative mutants of Shc in UT7 cells would allow us to answer this question.

Apart from Shc, many different cascades have been shown to link cytokine receptors to the Ras-MAPK pathway (34, 49). For instance, accumulation of activated Ras can result from

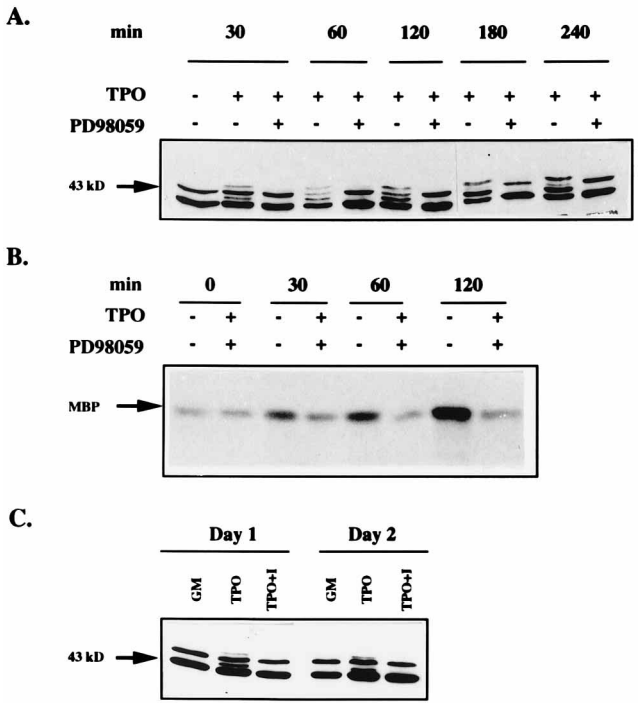

FIG. 8. Inhibition of ERK activation induced by TPO in UT7-mplWt cells by the MEK inhibitor PD98059. UT7-mplWt cells were pretreated for 1 h at 37°C with DMSO alone or 10 μM PD98059 (I) and then incubated with 400 U of TPO per ml, 2.5 ng of GM-CSF per ml, or medium alone for the indicated times at 37°C. ERK activation was assessed by electrophoretic shift (A and C) or kinase (B) assays.

enhancement of GDP-GTP exchange via receptor-linked PTP-1D phosphatase–GRB2–SOS complexes (32) or from reduction of hydrolysis of GTP by an action on Ras GTPase-activating protein (13, 34). Ras-independent pathways have also been described, involving, for example, direct activation of Raf by PKC (5, 6, 29). Mpl has been shown to bind PTP-1D and GRB2 (38), but no correlation with its capacity to activate MAPK was described. Although the capacity of TPO to activate PKC has, to our knowledge, not been described, it may be a good candidate for controlling ERK activation in UT7 cells. Indeed, phorbol esters are potent activators of megakaryocytic differentiation in UT7 cells as well as in other megakaryoblastic cell lines, and this function can be directly inhibited by PKC inhibitors (24, 30). On the other hand, the participation of Src in MAPK activation (18) would be worth examining in UT7 cells, since in hematopoietic cells, Src is mainly expressed in platelets and megakaryocytes (52). Finally, different forms of Raf could be used downstream of Mpl in BAF3 and UT7 cells. Notably, different B-Raf isoforms have been detected in these two cell types (17). Further studies will determine the pathways upstream of MEK that are triggered by TPO in UT7 cells and their respective contributions to controlling the level and kinetics of the signal.

Whatever the mechanism operating, control of the intensity and duration of the MAPK signal in UT7 cells seems to involve residues 71 to 94 of the cytoplasmic domain of Mpl, since the mplΔ3 mutant in which these residues were removed was unable to efficiently activate MAPK in this cell line. However, the capacity of mplΔ3 to transduce a low and transient activation of MAPK in UT7 cells suggests that distinct regions of the cytoplasmic domain of Mpl are contributing to mediate full MAPK activation in these cells. An Mpl mutant, in which the membrane-proximal region of Mpl (including box 1) has been deleted, cannot activate JAK2 kinase phosphorylation (44). This mutant was also unable to transduce MAPK activation (45), suggesting a requirement for tyrosine phosphorylation of Mpl in MAPK activation. The Δ3 region contains a tyrosine residue which could create a site for phosphotyrosine-binding sites present in many signaling mediators. However, mutational substitution of this tyrosine for a phenylalanine resulted in an Mpl mutant which was able to transduce both differentiation and strong, sustained activation of MAPK in UT7 cells (45). The region deleted in Δ3 does not contain any other amino acid or sequence described to play a role in protein-protein interactions and may therefore involve a new type of interaction. Alternatively, although the Δ3 deletion does not seem to affect the recruitment of Shc, we cannot rule out that conformational changes in the mplΔ3 mutant would affect binding of signaling molecules to nearby residues or sequences.

Very recently, several studies defined distinct Mpl receptor domains and signaling pathways involved in differentiation and proliferation functions in myeloid cells (1, 21, 37). It was reported that the Mpl C-terminal 20 amino acids and more precisely Y616 of this region is a critical site for both Shc recruitment to the receptor, Fos induction, and TPO-mediated differentiation and growth arrest in FDC/P2, M1, and WEHI3B-D$^+$ cells. Although MAPK activation was not tested in the studies mentioned above, this finding strongly suggests that this pathway may be involved in TPO-mediated differentiation signals in these systems as well. In none of these reports were Mpl mutants with an internal deletion equivalent to Δ3 tested. In M1 and WEHI3B-D$^+$ cells, TPO induces macrophage differentiation. It would be very interesting to study the amplitude and duration of MAPK activation by TPO (and the role of the Δ3 domain of Mpl in this activation) in these cellular models to determine whether the long-lived activation of MAPK is specifically linked to the physiological induction of megakaryocytic differentiation by TPO.

As opposed to differentiation, Mpl-mediated proliferation seems to be largely independent of MAPK activation, at least at optimal concentrations of TPO. Indeed in both BAF3 and UT7 cells, blocking the MAPK pathway with MEK inhibitor did not affect TPO-induced long-term cell growth. However, recent studies with BAF3 cells (1, 15) have shown that a deletion of the C-terminal domain of Mpl abolishing Shc activation increases the concentration of TPO required for maximal proliferation. This may suggest that although not essential, the MAPK pathway may make some contribution to the proliferative signal at suboptimal concentrations of TPO. In addition, the role of the MAPK pathway in TPO-mediated DNA synthesis and/or antiapoptotic responses should be reanalyzed in long-term culture in the absence of serum. Indeed, an elegant study has shown that these functions can be separately controlled by the GM-CSF receptor and that serum can provide Ras activation (28).

MAPKs translocate to the nucleus upon activation (53), where they elicit the phosphorylation of several different transcription factors, such as Fos and Jun, and thereby elicit their activation and gene induction (55). Sustained activation of MAPK has been shown to be a prerequisite for their nuclear translocation (53). Thus, the strong and long-lived activation of MAPK in UT7 cells may result in transcription of genes either of the megakaryocytic lineage or specifically involved in some processes of megakaryocytic maturation and growth arrest. On the other hand, ERK has been shown to induce serine phosphorylation and increase transcriptional activity of STAT1 (59). STAT3 and STAT5 are also phosphorylated on serine residues (3, 59) by yet unidentified kinases. In addition, recent studies indicated a critical role for STAT3 in IL-6-mediated differentiation (36, 61). TPO activates STAT1, -3, and -5 in various cell lines (1, 15, 21, 37, 38, 41), and experiments are currently under way to determine their role in induction of megakaryocytic genes.

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M. C. Rouyez and C. Boucheron contributed equally to this work.

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