Sustained Activation of the Extracellular Signal-regulated Kinase/Mitogen-activated Protein Kinase Pathway Is Required for Megakaryocytic Differentiation of K562 Cells*

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Frederick K. Racke‡, Kristine Lewandowska‡, Said Goueli§, and Adam N. Goldfarb‡¶

From the §Signal Transduction Division, Promega Corporation, Madison, Wisconsin 53711

The extracellular signal-regulated kinase (ERK), originally identified as a participant in mitogenic signaling, has recently been implicated in the signaling of cellular differentiation. To examine the role of the ERK/MAP kinase pathway in megakaryocytic differentiation of K562 cells, the effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) and bryostatin on ERK activation were determined. Both TPA and bryostatin are known to activate PKC but paradoxically have opposing effects on megakaryocytic differentiation. TPA, a differentiation inducer, caused sustained activation of ERK (>24 h), whereas bryostatin, a differentiation blocker, only transiently activated ERK (~6 h) and attenuated the activation of ERK by TPA. To confirm a requirement for sustained ERK activation for megakaryocytic differentiation, PD098059, a synthetic inhibitor of the MAP kinase kinase MEK1 was employed. Introduction of PD098059 at any time during the first 18 h of TPA treatment completely abrogated megakaryocytic differentiation of K562 cells. After 24 h of TPA treatment, introduction of PD098059 failed to block differentiation. Differentiation blockade by PD098059 occurred via inhibition of MEK because transfection of a constitutively active mutant of MEK2 could override the PD098059 blockade. Experiments with conditioned media suggested that sustained activation of the ERK/MAP kinase pathway promoted the autocrine secretion of megakaryocytic lineage determination factors.

The ERK/MAP pathway plays a well recognized role in relaying mitogenic signals from cell surface receptors to the nucleus to stimulate the G1/S transition (1–3). In particular, signaling via receptor tyrosine kinases leads to a characterized chain of biochemical events among which include the sequential activation of p21ras, Raf, MEK, ERK/MAPK, and the transcription factor Elk1 (2–4). The involvement of the ERK/MAPK pathway in mitogenic signaling suggests a potential role in oncogenesis, and accordingly, experimental expression of constitutively active mutants of MEK suffices to transform rodent fibroblasts (5). Conversely, dominant negative mutants of MEK revert ras- and src-mediated transformation of NIH 3T3 cells (6).

Paradoxically, the ERK/MAP kinase pathway also plays a well documented role in some systems of cellular differentiation. Nerve growth factor induction of neural differentiation in PC12 pheochromocytoma cells requires sustained activation and associated nuclear translocation of ERK/MAP kinase (4, 7). Microinjection of constitutively active mutants of MEK induces neurite extension in PC12 cells, and dominant negative mutants of MEK block NGF induction of neurite extension (6). Development of thymocytes from immature CD4+, CD8+ cells to intermediate CD4+, CD8+ cells also requires activation of ERK/MAP kinase (8), and positive thymic selection but not negative selection or T cell receptor-induced proliferation requires activation of the ERK/MAP kinase pathway (9). In Drosophila, cell fate determination of the R7 photoreceptor cells in the eye depends on the phosphorylation of the ets-family transcription factor Pointed by the kinase Rolled, a homolog of MAP kinase (10). Nevertheless, many types of cellular differentiation, including myogenesis and adipogenesis, appear to proceed in a MAPK-independent fashion (11). In hematopoietic differentiation, the role of the ERK/MAPK pathway has not yet been fully characterized.

Previous studies have shown that activation of protein kinase C (PKC) by the phorbol ester TPA induces megakaryocytic differentiation of the multipotent human cell line K562 (12–15). Interestingly, activation of PKC by the macrocyclic lactone bryostatin not only fails to induce megakaryocytic differentiation of K562 cells but actively blocks the differentiative effects of TPA (14, 16). The ERK/MAP kinase pathway, known to be activated by PKC and to be involved in some models of cellular differentiation, represents an appealing target for the opposing effects of TPA and bryostatin on K562 cells. Therefore, we examined whether TPA and bryostatin had differential effects on the activation of the ERK/MAP kinase pathway. Using two independent assay systems, the kinetics of ERK activation in response to TPA alone, bryostatin alone, and TPA plus bryostatin were characterized. TPA alone induced a sustained activation of ERK for >24 h. Bryostatin alone induced a transient activation of ERK lasting approximately 6 h. In combination, bryostatin attenuated the activation of ERK by TPA, with inactivation of ERK at 10–14 h post-initiation of stimulation. These data suggested a model similar to that in PC12 cells, namely that sustained activation of the ERK/MAP kinase pathway is necessary for megakaryocytic differentiation of K562 cells (4, 7).

As independent confirmation of this model, we employed a selective inhibitor of the ERK/MAP kinase pathway, the synthetic compound PD098059, which prevents activation of the MAP kinase kinase MEK1 by upstream kinases (17, 18). Both MEK1 and MEK2 may activate the ERKs, but MEK1 appears...
to be the primary MAP kinase kinase element involved in signaling via p21^{ras} (19). Treatment of K562 cells with PD098059 completely abrogated TPA induction of the megakaryocytic surface marker CD41. Strikingly, delayed addition of PD098059 up to 18 h post-initiation of TPA still abrogated cellular differentiation efficiently. As confirmation that PD098059 exerted its effects via the ERK/MAP kinase pathway, expression in K562 cells of a constitutively active mutant of MEK2 overrode inhibition by PD098059. These data therefore confirmed that sustained activation of the ERK/MAP kinase pathway for >18 h is required for megakaryocytic differentiation.

Whereas PD098059 effectively blocked TPA induction of megakaryocytic differentiation, conditioned media from TPA-induced K562 cells induced megakaryocytic differentiation resistant to PD098059 inhibition. The effects of PD098059 appeared to be on the actual media conditioning process, in that conditioned media from cells treated with TPA plus PD098059 failed to induce differentiation. Therefore we conclude that the sustained activation of the ERK/MAP kinase pathway appears to participate in the establishment of an autocrine signaling loop that is required for megakaryocytic differentiation of K562 cells.

**MATERIALS AND METHODS**

**Assays for MAP Kinase Activation**—Immunoblots of whole cell extracts with antibodies specific for activated ERK used the anti-Active™ MAPK pAb (Promega Corp., Madison, WI), which recognizes the dual phosphorylated forms of p42 ERK2 and p44 ERK1. Whole cell extracts for immunoblots were obtained by agitating 1 × 106 cells for 20 min in 1 ml of ice-cold radioimmune precipitation buffer 150 (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 0.2 unit/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 0.1 mM sodium meta-vanadate). 100 μg of protein/sample (as determined by the Bradford assay) were separated by 12% SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting as described previously (20). For immunoblotting, anti-Active™ MAPK pAb was used at a final concentration of 25 ng/ml. Three independent immunoblot time course experiments were performed. Densitometry was performed by scanning developed membranes with a MicroTek flat bed scanner and applying the NIH Image software on a Macintosh platform for signal quantitation. All signals were normalized to those obtained with uninduced K562 cells.

For immunoprecipitation kinase (IP-kinase) assays, 5 × 106 cells/time point were extracted for 20 min with 2 ml of ice-cold radioimmune precipitation buffer 150/IPK buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate with protease and phosphatase inhibitors as in radioimmune precipitation buffer 150). To 1 ml of extract (2.5 × 106 cellular equivalents), 1 μg of rabbit anti-ERK (C-14, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added followed by 1 h of rotation at 4°C. Immune complexes were collected on 20 μl of protein A-agarose beads preblocked with bovine serum albumin. After four washes in 1 ml of ice-cold radioimmune precipitation buffer 150/IPK buffer, the beads were resuspended in 50 μl of kinase reaction mixture (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 150 mM NaCl, 0.1% 2-mercaptoethanol, 1 mg/ml bovine serum albumin, 300 ng/ml myelin basic protein, 50 μM cold ATP, 100 μCi/ml [γ-32P]ATP, 2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride). After a 20-min incubation at 30°C, samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by electroblotting and autoradiography. Two independent IP-kinase time course experiments were performed. For direct signal quantitation, electroblotted gels were scanned with a Packard InstantImager™ electronic autoradiography system. The CD8 expression plasmid pCD8-REP7 was kindly provided by Dr. Michael J. Weber (Department of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA). The CD8 expression plasmid pCD8-REP7 was kindly provided by Dr. Mark Tykocinski (Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH). Transfections were performed with the liposomal formulation Dotap, following the guidelines of the manufacturer (Boehringer-Mannheim).

**RESULTS**

Sustained Activation of ERK Correlates with Induction of Megakaryocytic Differentiation—K562 cells treated with TPA recapitulate several of the events of early megakaryocytic differentiation including acquisition of megakaryocytic surface markers, production of megakaryocytic cytokines, cell cycle arrest, cellular enlargement, and development of polyploid nuclei (12–15). A broad range of TPA concentrations (1 nm–1 μM) may induce differentiation, as judged by up-regulation of the platelet surface glycoprotein IIb/IIIa (CD41 antigen), an early marker of megakaryocytic differentiation. The onset of CD41 expression occurs approximately 24 h after continuous cellular stimulation with TPA, indicating a lag between initiation of stimulus and commencement of differentiation. By contrast, bryostatin does not induce any aspect of megakaryocytic differentiation in K562 cells and, in fact, effectively blocks TPA induction of differentiation (14). The opposing effects of bryostatin and TPA on megakaryocytic differentiation are paradoxical, given that both compounds potently activate PKC.

To study the effects on the ERK/MAP kinase pathway of TPA and bryostatin alone and in combination, K562 cells were analyzed by two independent techniques, immunoblotting with an antibody specific for activated ERK kinases and IP-kinase...
assays with an anti-ERK antibody. Fig. 1A shows representative time course immunoblots of K562 cellular extracts with an antibody specific for dual phosphorylated ERK kinases. Fig. 1B graphically depicts the pooled immunoblot data from three independent time course experiments. All three stimuli (TPA, bryostatin, and TPA + bryostatin) caused an initial activation of the ERK kinases of 5–10-fold over uninduced cells. However, because 0.25% Me₂SO alone did not block megakaryocytic differentiation (Fig. 3A), the final Me₂SO concentration was 0.25%. The percentage of CD41 positive cells was determined in each panel. C and D, transiently transfected MEK2 overrides the effects of the MEK1 inhibitor PD098059. In panel C, cells treated with TPA and PD098059 were transfected with the vector pCMV-ΔN-MEK2-S222/226D, which encodes a constitutively active mutant of MEK2. In panel D, cells treated with TPA and PD098059 have been transfected with pCMV-MEK2-KA, which encodes a kinase-dead mutant of MEK2. At 24 h of induction, cells were analyzed by FACS for CD41 expression. To control for transfection efficiency, cells were cotransfected with an expression plasmid for the surface protein CD8, pCD8-REP76, and the percentage of CD8-positive cells was determined by FACS. 46% for the cells in panel C and 55% for the cells in panel D. The transient transfection experiment represented in panels C and D was performed a total of three times, with similar results on each occasion.

A Specific MEK1 Inhibitor Blocks Megakaryocytic Differentiation through Inhibition of the ERK/ MAP Kinase Pathway—The synthetic compound PD098059 inhibits the ERK/MAP kinase pathway by preventing the activation of MEK1 by c-Raf (17, 18). Extensive testing both in vitro and in vivo has shown no inhibition by PD098059 of any other known kinases or kinase pathways (18). The biological efficacy of this compound has previously been demonstrated by its ability to block NGF-induced differentiation in PC12 cells without any cytotoxicity (11). The in vitro IC₅₀ for MEK1 inhibition by PD098059 is 2–7 μM; the in vitro IC₅₀ for MEK2 inhibition is 50 μM. As shown in Figs. 3, A and B, PD098059, when present at 25 μM during induction, completely abrogates TPA induction of megakaryocytic differentiation, as assessed by CD41 expression. In addition to blocking CD41 expression, PD098059 also blocks cellular enlargement and partially reverses cell cycle arrest (data not shown). Furthermore, the inhibition was not due to non-specific cytotoxicity, as the compound actually enhanced cell viability during TPA treatment (data not shown). Nor were the observed effects due to the presence of 0.25% Me₂SO carrier because 0.25% Me₂SO alone did not block megakaryocytic differentiation (Fig. 3A). To confirm that the effects of PD098059 were mediated via the ERK/MAP kinase pathway, we performed a reversal experiment, taking advantage of the relatively high IC₅₀ for MEK2. In particular, transient transfection of a constitutive active MEK2 mutant ΔN-MEK2-S222/226D reversed the inhibitory effect of PD098059 on CD41 expression, whereas transfection of a kinase-dead MEK2 mutant MEK2-KA failed to reverse the blockade of CD41 expression (Figs. 3, C and D, respectively). In Fig. 4, independent evidence for the involvement of the ERK/MAPK pathway in megakaryocytic differentiation is provided; transient transfection of K562 cells with the kinase-dead mutant MEK2-KA partially inhibited TPA induction of CD41 expression (Fig. 4C), whereas transfection with the parent vector and with the constitutive active mutant ΔN-MEK2-S222/226D had no inhibitory effects on CD41 induction (Figs. 4, A and B, respectively).

Sustained ERK Activation Is Required for Megakaryocytic Differentiation—The data obtained with TPA and bryostatin treatment of K562 cells (Figs. 1 and 2) suggested that sustained activation of the ERK/MAP kinase pathway might be...
required for megakaryocytic differentiation. To address this issue directly, PD098059 was added to cells at various time points during the course of differentiation induction. All samples were analyzed for expression of CD41 after a total of 48 h of TPA induction. Fig. 5 shows that addition of PD098059 as late as 18 h after initiating induction still almost completely blocked megakaryocytic differentiation. However, addition of PD098059 at 24 h of induction failed to block megakaryocytic differentiation.

ERK-independent Induction of Differentiation by Conditioned Media—The requirement for sustained ERK activation for >18 h raised the possibility that the ERK/MAP kinase pathway might participate in establishment of an autocrine signaling loop. To examine this possibility, conditioned supernatants from K562 cells treated with TPA were applied to fresh cells. To ensure that carry-over of TPA in the conditioned media did not directly induce cellular differentiation, 25 μM PD098059 was added to the conditioned media at the time of application to fresh cells. Surprisingly, PD098059 failed to block differentiation induction by conditioned media (Fig. 6A). However, if 25 μM PD098059 was included during the initial TPA treatment step, the resultant conditioned media failed to induce differentiation (Fig. 6B). Similar results were obtained with extensively dialyzed, serum-free conditioned media, ruling out possible effects of cellular metabolite accumulation or simple nutrient depletion (data not shown).

DISCUSSION

The ERK/MAP kinase pathway regulates two mutually antagonistic processes, cellular proliferation and cellular differentiation. Two explanations may resolve this apparent paradox. First, the effects of ERK activation may be cell-type specific as suggested by the different effects of ERK activation in NIH-3T3 cells versus PC12 cells, the former undergoing transformation and the latter undergoing differentiation (6). Second, the duration and subcellular localization of ERK activation may influence the cellular response (4). In particular, epidermal growth factor, which causes a transient cytoplasmic activation of ERK, fails to induce differentiation of PC12 cells,
whereas NGF, which causes a sustained nuclear activation of ERK, successfully induces differentiation of PC12 cells (7).

The antagonistic effects of TPA and bryostatin on K562 cell differentiation show some parallels with the effects of NGF and epidermal growth factor on PC12 cell differentiation. In particular, both TPA and bryostatin cause the activation of ERK in K562 cells, presumably through their activation of PKC; importantly, the two agents show markedly different kinetics in their activation of ERK, with TPA causing sustained activation beyond 24 h and bryostatin causing transient ERK activation with a return to near base line at approximately 10 h. In contrast to observations in the PC12 system, the subcellular localization of activated ERK does not differ between TPA and bryostatin-treated K562 cells: both treatments are associated with a pancellular (nuclear and cytoplasmic) distribution of activated ERK. The ability of bryostatin to actively suppress megakaryocytic induction by TPA, previously unexplained, correlates well with the ability of bryostatin to alter the kinetics of ERK activation by TPA. In particular, bryostatin causes a delayed down-regulation in the ERK activation by TPA, beginning at 6–10 h of induction. By approximately 14 h of induction with bryostatin + TPA, ERK activation returned to base line levels. Our experiments with the MEK1 inhibitor, PD098059, confirm that down-regulation of ERK activation as late as 18 h into induction suffices to block megakaryocytic differentiation. The exact mechanism by which bryostatin causes delayed down-regulation of TPA-induced ERK activation remains unknown but might relate to differential effects of TPA and bryostatin on phosphatase activation.

The requirement of sustained ERK activation for megakaryocytic differentiation appears to represent an interesting threshold effect. Studies on Swiss 3T3 cells show that 50 μM PD098059 only partially inhibits the TPA activation of ERK by approximately 70% or 3-fold (18). The 25 μM levels of PD098059 successfully employed in our experiments are therefore predicted to diminish ERK activation by even less, only 2-fold. It is therefore likely that TPA stimulation of K562 cells achieves an activation of ERK only slightly above the threshold required for induction of megakaryocytic differentiation. If TPA stimulated ERK activation far above the threshold required for differentiation, PD098059 would most likely prove ineffective as an inhibitor.

An autocrine model for the megakaryocytic differentiation of K562 cells explains many of the properties of this experimental system: 1) the ability of conditioned media to induce differentiation even in the presence of PD098059, 2) the 24-h lag phase between initiation of TPA treatment and the onset of CD41 expression, 3) the absence of classic TPA response elements in the promoters for the genes constituting CD41, promoters that nevertheless display TPA inducibility (21, 22), 4) the appearance on anti-phosphotyrosine immunoblots of multiple new bands at 24 h of TPA stimulation, and 5) the requirement for sustained activation of the ERK/MAP kinase pathway for >18 h. The requirement of sustained activation of ERK above a critical threshold suggests a cumulative phenomenon in which ERK signaling contributes to the accumulation of a mediator, in this case secreted factors, which eventually achieves levels sufficient to trigger megakaryocytic differentiation of K562 cells. We therefore propose a two-step model to describe the early phases of megakaryocytic differentiation. In the earliest lineage commitment phase, sustained ERK activation contributes to the secretion of autocrine factors. In a secondary phase, the cells respond to these factors by initiating a program of terminal differentiation.

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