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Megakaryocytic Differentiation Induced by Constitutive Activation of Mitogen-Activated Protein Kinase Kinase

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The K562 erythroleukemia cell line was used to study the molecular mechanisms regulating lineage commitment of hematopoietic stem cells. Phorbol esters, which initiate megakaryocyte differentiation in this cell line, caused a rapid increase in extracellular-signal-regulated kinase (ERK), which remained elevated for 2 h and returned to near-basal levels by 24 h. In the absence of extracellular stimuli, ERK could be activated by expression of constitutively active mutants of mitogen-activated protein (MAP) kinase kinase (MKK), resulting in cell adhesion and spreading, increased cell size, inhibition of cell growth, and induction of the platelet-specific integrin $\alpha_{IIb}\beta_3$, all hallmarks of megakaryocytic differentiation. In contrast, expression of wild-type MKK had little effect. In addition, constitutively active MKK suppressed the expression of an erythroid marker, α-globin, indicating the ability to suppress cellular responses necessary for alternative cell lineages. The MKK inhibitor PD98059 blocked MKK/ERK activation and cellular responses to phorbol ester, demonstrating that activation of MKK is necessary and sufficient to induce a differentiation program along the megakaryocyte lineage. Thus, the MAP kinase cascade, which promotes cell growth and proliferation in many cell types, instead inhibits cell proliferation and initiates lineage-specific differentiation in K562 cells, establishing a model system to investigate the mechanisms by which this signal transduction pathway specifies cell fate and developmental processes.

It is now well established that the mitogen-activated protein (MAP) kinase cascade is a key regulator of mammalian cell proliferation (44). This pathway includes the MAP kinases extracellular-signal-regulated kinase 1 (ERK1) and ERK2, which are phosphorylated and activated by MAP kinase kinase 1 (MKK1) and MKK2 (44). ERKs are able to phosphorylate and activate the MAP kinase-activated protein (MAPKAP) kinases, including $pp90^{rsk}$ (47), MAPKAP kinase 2 (46), and 3pK (45). MKK1 and MKK2 can be phosphorylated and activated by any of three protein kinases, Raf-1, MEK kinase, and Mos, and thus represent convergence points for diverse signalling pathways triggered upon cell surface receptor activation. Downstream targets of the MAP kinase cascade include several transcription factors which may be regulated by direct phosphorylation by the ERKs or MAPKAP kinases (9, 13, 20, 32, 50, 56). Thus, the mitogen-activated MAP kinase cascade is a key mechanism for the control of transcription by extracellular signals.

Several observations underscore the essential role for the MAP kinase pathway in cell transformation and cell cycle regulation. Signalling components further upstream, including receptor tyrosine kinases, Src, Ras, Raf-1, and the G_α subunit of heterotrimeric G proteins, have been found in mutant oncogenic forms in transformed cells (3). Furthermore, expression of constitutively activated MKK1 and MKK2 mutants initiates S-phase entry in quiescent cells and induces cellular transformation (6, 11, 26). Conversely, inhibition of the MAP kinase pathway with dominant negative mutants of MKK blocks v-*ras*- or v-*mos*-dependent cell transformation (11, 31), and antisense or dominant negative inhibition of ERK inhibits cell growth and growth factor-stimulated gene transcription (18, 20, 33).

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In contrast to its function in regulating cell growth, the MAP kinase pathway has been shown to play an important role in regulating embryonic development and cell differentiation. Activation of MKK or ERK is essential for vulval development in *Caenorhabditis elegans* (48), photoreceptor cell specification and anterior-posterior body patterning in *Drosophila melanogaster* (36), mesoderm induction during *Xenopus laevis* embryo development (52), and positive T-cell selection (1). One approach to understanding these processes at a mechanistic level is through manipulation of cultured cells which are transformed at incomplete stages of differentiation. Many such cells proliferate in culture but undergo cell cycle arrest and differentiation in response to appropriate stimuli (28). So far, the only example of a differentiation program induced in cultured cells by the MAP kinase cascade occurs in the rat pheochromocytoma (PC12) line, where prolonged activation of ERK in response to treatment with nerve growth factor (NGF) leads to neuronal differentiation (11, 27, 39, 51). Comparative studies with other cell lines are needed in order to test the generality of conclusions reached from studies with these cells.

K562 is a human erythroleukemia cell line derived from a patient with chronic myelogenous leukemia (22). These cells are pluripotent in that they are able to differentiate along a megakaryocytic, erythroid, or, to a lesser extent, monocytic lineage (2). Hemin, aphidicolin, or 1-β-D-arabinofuranosylcytosine commits K562 cells towards an erythroid lineage (14, 29, 41, 42). In contrast, phorbol 12-myristate 13-acetate (PMA) induces differentiation into megakaryocytes, with concomitant loss of monocyte- and erythroid cell-specific markers (7, 49). Phorbol esters stimulate the MAP kinase pathway in many cells, through protein kinase C-dependent as well as protein kinase C-independent mechanisms (30). This observation raises the possibility that ERK activation in K562 cells is involved in specifying the megakaryocyte lineage. In this study, we demonstrate a key role of the mitogen-activated MAP ki-

nase pathway during megakaryocyte differentiation in K562 cells, establishing a model system for examining the effects of mitogenic signal transduction pathways on cell proliferation versus differentiation along myeloid lineages.

MATERIALS AND METHODS

Cell culture. Human erythroleukemia cell lines K562 and HEL were obtained from the American Type Culture Collection. Cells were maintained in spinner cultures at a density of 5×10^5 cells/ml in RPMI medium supplemented with 10% (vol/vol) fetal calf serum (Gibco/BRL), 100 U of penicillin per ml and 100 U of streptomycin per ml.

Vectors. Plasmids for mammalian cell expression of hemagglutinin (HA)-tagged wild-type and catalytically inactive (K97M) MKK1 and MKK2 have been described elsewhere (24, 26). Constitutively active mutants have activities greater than that of the wild type to the following extents: MKK1-11A/55B (S218E/S222D), 65-fold; MKK1-X7 (ΔN3 [deleted residues 32 to 51]/S222D), 270-fold; MKK1-R4F (ΔN3/S218/S222D), 490-fold; MKK1-G1C (ΔN4 [deleted residues 44 to 51]/S218E/S222D), 530-fold; and MKK2-KW71 (ΔN4 [deleted residues 44 to 51]/S218D/S222D), 2,100-fold (24–26). Constructs for mammalian expression of HA-tagged wild-type ERK2 in pCEP4 and for bacterial expression of ERK2 (K52R) were gifts of Melanie Cobb. A construct for mammalian expression of v-Raf (3611-MSV) was a gift of Peter Shaw. A construct expressing the green fluorescent protein was a gift of Roger Tsien.

Cell treatment and extraction. Aliquots (10 to 20 ml) of exponentially growing cells (2.5×10^5 cells/ml, passed into fresh media 4 to 6 h prior to PMA addition) were divided into separate 10-cm-diameter petri dishes and treated with PMA (final concentration, 50 nM; 0.1% [vol/vol; final concentration] ethanol) or control treated with ethanol. At various times, cells were harvested by centrifugation ($2,000 \times g$, 5 min, 4°C) and washed twice with ice-cold phosphate-buffered saline (PBS), and pellets were frozen at −80°C. For time points >36 h, cells were centrifuged and resuspended each day in fresh media containing 50 nM PMA. For the 5-min time point, cells were harvested immediately following PMA addition. In experiments utilizing the PD98059 MKK inhibitor, cells were treated with PD98059 (Calbiochem) dissolved in dimethyl sulfoxide (0.1%, vol/vol [final concentration]) at various times either prior to or after the addition of PMA (50 nM).

Cell transfection. K562 cells were transfected by electroporation (960 μF; 220 V) using a Bio-Rad Gene Pulser electroporator. Cells (5×10^6) were washed, resuspended in 0.4 ml of Optimem (Gibco-BRL) supplemented with 5% fetal calf serum without antibiotics, and placed in a 0.4-cm gap electroporation cuvette (BTX). Plasmid DNA (35 μg) was added, and after a brief incubation at room temperature the cells were subjected to a single electric pulse followed by dilution to 2.5×10^5 cells/ml with RPMI medium containing 10% fetal calf serum and antibiotics. Transfection efficiencies ranging from 15 to 25% were measured in parallel electroporations for each experiment, assessed by expression of cytomegalovirus β-galactosidase (Clontech) or green fluorescent protein. For the 96-h time points described below for flow cytometry or RNA analyses, cells were centrifuged and resuspended in fresh medium each day.

Protein kinase reactions and immunoblotting. Frozen cell pellets were thawed in 0.25 ml of extract buffer (50 mM β-glycerophosphate, pH 7.4; 1 mM EGTA; 1.0 mM sodium vanadate; 1 mM dithiothreitol; 0.1 mM sodium molybdate; 10 μg of aprotinin per ml; 2 μg of pepstatin A per ml; 10 μg of leupeptin per ml; 1 mM benzamidine; and 0.01% Triton X-100) and sonicated for 5 s (Branson needle probe sonicator). Lysates were centrifuged ($17,000 \times g$, 2 min), and supernatants (30 μl) were immunoprecipitated with polyclonal antibodies (1 μg) recognizing ERK2 (SC154; Santa Cruz) or monoclonal antibodies (1 μg) recognizing the HA tag (12CA5; Babco). Protein A-Sepharose (10 μl; Pharmacia) was added after 1 h, and the incubation was continued for 1 h with occasional agitation. Protein A-Sepharose immunocomplexes were pelleted by brief centrifugation and washed twice with buffer A (25 mM HEPES, pH 7.4; 25 mM β-glycerophosphate; 2.5 mM $MgCl_2$; 2 mM dithiothreitol; 0.2 mM sodium vanadate), and reactions were initiated by addition of substrate (final concentration, 0.15 mg/ml) and ATP (30 μM with 10 μCi of [γ-^{32}P]ATP) in a final volume of 30 μl. The substrate for immunoprecipitated ERK2 was myelin basic protein (Sigma), and the substrate for MKK was ERK2(K52R) purified from a bacterial expression system by Ni^{2+}-nitriloacetic acid-agarose chromatography as described previously (40). After 5 min at 30°C, reactions were terminated by addition of 40 μl of 2× Laemmli loading buffer. The samples were boiled, separated by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE) (acrylamide-bisacrylamide, 29:0.1), and transferred to Immobilon-P membranes (Millipore). Membranes were probed by Western blotting using either 12CA5 or SC154 antibody and developed with alkaline phosphatase-coupled goat anti-mouse antibodies (Promega). Kinase activities were then quantified by phosphorimager analysis of ^{32}P-labelled substrates on the blots.

For analysis of α-globin expression, cells were transiently transfected with MKK as described above. At 24 h posttransfection, cells were resuspended in fresh RPMI medium–10% fetal bovine serum in the presence or absence of 20 μM hemin (Sigma). Untransfected cells were also treated with 50 nM PMA or 20 μM hemin at this time. Cells were harvested 48 h later and processed as total cellular extracts as described above. Total cellular protein (30 μg) from each treatment was separated by SDS–15% PAGE, transferred to Immobilon-P membranes, and probed with monoclonal antibody 12CA5 (recognizing HA-tagged MKK) or monoclonal antibody 3G12, raised against the α-globin subunit of human hemoglobin (a gift of Michael Schick). Immunoreactivity was detected by enhanced chemiluminescence (Amersham) using donkey anti-mouse antibody-conjugated horseradish peroxidase (Jackson Immunoresearch).

RNA analysis. Total RNA from HEL or K562 cells (1×10^6 to 2×10^6) was extracted by a modification of the protocol of Chomczynski (10). Unwashed cell pellets were lysed in 1 ml of Triazol (Gibco-BRL), incubated for 5 min at room temperature, and mixed with 0.2 ml of chloroform. Organic and aqueous phases were partitioned by centrifugation ($17,000 \times g$, 1 min, 4°C), and the aqueous (upper) layer (0.5 ml) was transferred to a 1.5-ml tube. RNA from this phase was precipitated with isopropanol, washed with cold 70% ethanol, dried, and resuspended in 10 mM Tris (pH 8)–1 mM $MgCl_2$–50 mM KCl (50 μl). RNA samples were then treated with RNase-free DNase I (15 min, 37°C), heat inactivated (15 min, 65°C), phenol-chloroform extracted, and precipitated by addition of cold ethanol. Pellets were recovered by centrifugation and resuspended in 10 mM Tris (pH 8.0)–1 mM EDTA–50 U of RNase inhibitor (Gibco-BRL) per ml–1 mM dithiothreitol prepared in diethyl pyrocarbonate-treated water. The concentration and purity of each sample were assessed spectrophotometrically, and integrity was examined by electrophoresis on a 1% agarose–Tris-acetate-EDTA gel.

In order to prepare constructs for T7 RNA polymerase synthesis of antisense RNA, total HEL cell RNA (2 μg) was reverse transcribed with 1 U of Moloney murine leukemia virus reverse transcriptase and 1 mM random hexamers in a 25-μl reaction mixture with 1 mM nucleoside triphosphates, 10 mM Tris (pH 8.3), 50 mM KCl, 1 mM dithiothreitol, and 8 mM $MgCl_2$. The resulting cDNA pool was amplified with 200 μM nucleoside triphosphates–10 mM Tris (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–0.5 U of *Taq* polymerase (Perkin-Elmer)–1 μM sense-antisense primers specific for human β-actin (5′ sense, TGACGGG GTCACCCACACTGTGCCCATCTA; 3′ antisense, CTAGAAGCATTTGCG GTGGACGATGGAGGG), human integrin α_{IIb} (sense, AGCTACTGGTGCA AGCTTCAC; antisense, GCGCCCCGGGGCAGGTGCACG), or human integrin β_3 (sense, GCCTCTGGGCTCACCTCGCTG; antisense, CTGGGATAGC TTCTCAGTCATCAGCCC). The PCR mixture was separated on a 1% agarose–Tris-acetate-EDTA gel, and major reaction products were eluted from gel fragments and subcloned into the T-tailed Novablue vector (Novagen). The authenticity of the insert was determined by sequencing from the T7 promoter site.

To quantify mRNA, total K562 cell RNA (2 μg) was reverse transcribed as described above. Control reactions were run in the absence of reverse transcriptase. The reverse transcriptase products (1 to 5 μl) were added to 50 μl of PCR mixture containing 1 U of *Taq* polymerase and 1 μM primers for human β-actin (IDX) or integrin α_{IIb} or β_3 (see above). Each PCR was run for 35 to 40 cycles and terminated by addition of 6× loading buffer (10 μl), and 35 μl was separated on 1% agarose–Tris-acetate-EDTA gels. Gels were incubated with 1 M NaOH–0.5 M NaCl (15 min) and neutralized with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 15-min washes), and separated products were transferred in 10× SSC onto Hybond-N membranes (Amersham). Blots were prehybridized and probed with T7 RNA polymerase γ-^{32}P-labelled antisense transcripts (10^7 cpm/μg) for β-actin, integrin α_{IIb}, or integrin β_3 in prehybridization solution (50% deionized formamide, 6× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA {pH 7.7}], 5× Denhardt's solution, 0.1% SDS, 100 μg of sheared denatured salmon sperm DNA per ml) at 42°C. Blots were then washed with 1× SSPE–0.5% SDS at 68°C (two 15-min washes) and exposed and quantified by phosphorimager analysis. The reaction product sizes for each marker were 661 bp (β-actin), 597 bp (integrin α_{IIb}), and 801 bp (integrin β_3).

Immunofluorescence. K562 cells treated with PMA or transfected with various DNA constructs were plated onto sterile glass coverslips in 100-mm-diameter petri dishes. After 72 h, coverslips were gently washed in warmed PBS and fixed in cold methanol-acetone (1:1, vol/vol). Coverslips were washed twice with PBS and incubated with gentle shaking for 30 min at 30°C with 1 μg of 12CA5 per ml in PBS-TB (10 mg of bovine serum albumin per ml, 0.5% Tween 20 in PBS). Coverslips were washed three times with PBS-TB and incubated for 30 min at 30°C with 5 μg of rhodamine-conjugated donkey anti-mouse antibody (Jackson Immunoresearch) per ml in PBS-TB. Coverslips were washed three times with PBS-TB and once in PBS and then incubated with 1 μg diamidino-2-phenylindole (DAPI) in PBS. After a final wash with PBS, coverslips were mounted onto slides in Mowiol (Sigma). Cells were visualized and photographed on a Zeiss Axiophot fluorescence microscope.

For ERK2 localization studies, untransfected cells or cells transfected with HA-tagged wild-type ERK2 under control of the cytomegalovirus promoter were treated with 50 nM PMA. Aliquots of cells were harvested, washed once with PBS at room temperature, and resuspended in PBS. Approximately 10^4 cells were centrifuged onto coverslips at $150 \times g$ for 5 min in a Cytospin 3 (Shandon Lipshaw). The cells were then fixed in neutral buffered 10% formalin (Sigma) for 5 min at room temperature and washed twice in PBS. Coverslips were blocked with TBS-TB, incubated for 4 h with either 5 μg of monoclonal anti-ERK2 (D2; Santa Cruz) per ml or 1 μg of 12CA5 per ml, washed three times in TBS-TB, and incubated for 2 h with 5 μg of CY3-conjugated donkey anti-mouse antibody (Jackson Immunoresearch) per ml. After three washes with TBS-TB, coverslips were briefly incubated with DAPI and mounted in Mowiol.

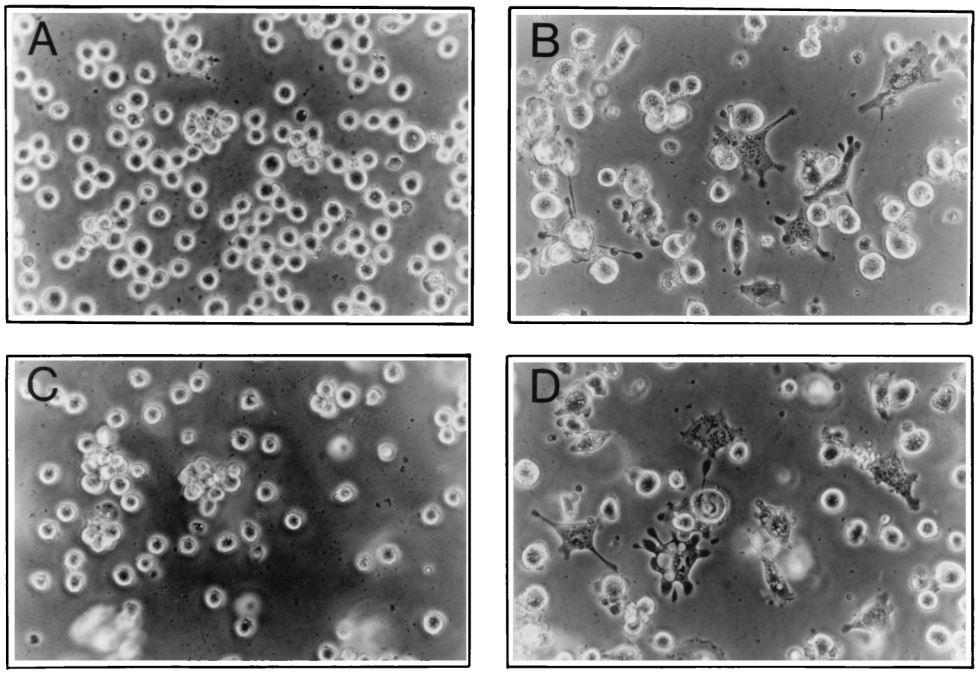

FIG. 1. Adhesion and spreading of K562 cells following PMA treatment or expression of MKK1. Cells in petri dishes were visualized by light microscopy. (A) Untreated cells; (B) cells treated with 50 nM PMA for 48 h, (C) cells transiently transfected with wild-type MKK1 for 48 h; (D) cells transiently transfected with constitutively active MKK1-R4F for 48 h. A significant number of cells transfected with MKK1-R4F were adherent to petri dishes, whereas cells transfected with wild-type MKK1 were not.

Flow cytometric analysis. Cells were harvested after treatment with PMA or electroporation, washed in PBS with 10 mg of bovine serum albumin per ml (PBS-B), and blocked for 1 h. Approximately 5×10^4 cells were incubated at 30°C in 200 μl of 20-μg/ml 7E3, a monoclonal antibody recognizing the integrin $\alpha_{IIb}\beta_3$ complex (a gift of Barry Coller). Cells were washed in PBS-B and incubated for 2 h with 10 μg of phycoerythrin-conjugated donkey anti-mouse antibody (Jackson Immunoresearch) per ml in PBS-B. Cells were washed in PBS-B, resuspended in PBS with 1% glycerol, and analyzed immediately or left overnight at 4°C and analyzed the following day. Flow cytometric analysis was carried out on a Becton Dickinson FACScan analyzer, and data were analyzed with the LYSIS BD program (Becton Dickinson).

RESULTS

Effects of constitutively active MKK mutants on K562 cell morphology. In order to test the effect of the MAP kinase cascade on K562 cell differentiation, constitutively activated MKK1 and MKK2 mutants (24–26) were expressed in these cells by transient transfection. Effects of wild-type and mutant MKK expression were compared to effects of PMA treatment. Following PMA treatment, K562 cells, which normally grow in suspension (Fig. 1A), gave rise to adherent cells with pseudopodia, enlarged nuclei, and spreading (Fig. 1B) similar to normal megakaryocytes (7, 49). By 12 h, adhesion and spreading could be observed in about 10% of cells, increasing to 25% of total cells after 96 h (data not shown). Overexpression of wild-type MKK1 did not alter the morphology of transfected K562 cells (Fig. 1C). However, cells transfected with the constitutively active mutant MKK1-R4F (ΔN3/S218E/S222D) assumed a morphology resembling that of the PMA-stimulated population (Fig. 1D). Similar effects were observed upon expression of the constitutively active MKK1-11A/55B (S218E/S222D), MKK1-G1C (ΔN4/S218E/S222D), MKK2-KW71 (ΔN4/S222D/S226D), or, to a lesser extent, v-Raf (data not shown). Under the conditions used, transfection efficiencies ranged from 15 to 25%. These data demonstrate that activation of MKK in K562 cells mimics morphological responses characteristic of a transition to the megakaryocytic lineage.

Expression of constitutively active MKK in a few cells may lead to induction of an autocrine factor capable of mediating adhesion and spreading in surrounding cells. To test this possibility, we examined the expression of HA-tagged MKK in cells that had differentiated after transfection. Cells were transfected with wild-type or constitutively active MKK, plated onto coverslips, and probed for MKK expression by using anti-HA antibody (Fig. 2). Antigen was detected in many but not all cells transfected with wild-type MKK1 (Fig. 2A to C) or MKK2 (Fig. 2G to I). However, high levels of anti-HA staining were observed in all cells that acquired an adherent and spread morphology following transfection with constitutively active MKK1 (Fig. 2D to F) or MKK2 (Fig. 2J to L). No significant cross-reactivity was observed in untransfected cells (Fig. 2M to O). Furthermore, conditioned medium from cells expressing constitutively active MKK did not promote adhesion of untransfected K562 cells (data not shown). The results indicate that active MKK mutants induce differentiation only in the transfected cells.

Attempts were made to produce stable cell lines expressing wild-type or constitutively active MKK1 or MKK2, by passaging cells in the presence of hygromycin (0.5 mg/ml) following electroporation (the MKK plasmid constructs express the hygromycin resistance gene under a thymidine kinase promoter). Cells transfected with constitutively active MKK1 (R4F or G1C) or MKK2 (KW71) stopped proliferating and became adherent within 1 to 2 weeks of selection, followed by cell lysis (data not shown). Similar effects were observed in nontransfected cells maintained for 2 weeks in the presence of phorbol ester (50 nM). In contrast, cells transfected with control vector or with wild-type MKK1 or MKK2 continued to proliferate and could be maintained as stable cell lines (data not shown).

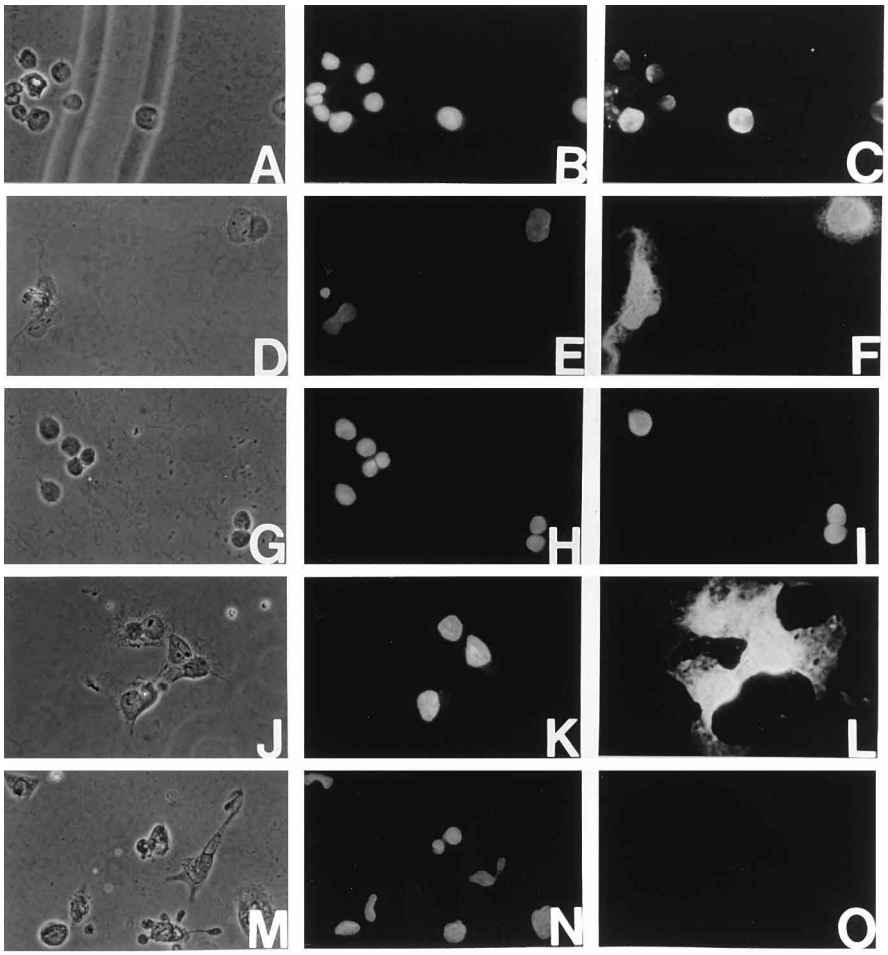

FIG. 2. Indirect immunofluorescence of K562 cells following MKK1 or MKK2 expression. Cells adhering to glass coverslips after 48 h of treatment were visualized by light microscopy (A, D, G, J, and M), immunofluorescent DAPI staining of nuclei (B, E, H, K, and N), or immunofluorescent staining of HA-tagged MKK (C, F, I, L, and O). (A to C) Cells transfected with wild-type MKK1; (D to F) cells transfected with constitutively active MKK1-G1C; (G to I) cells transfected with wild-type MKK2; (J to L) cells transfected with constitutively active MKK2-KW71; (M to O) untransfected cells treated with 50 nM PMA. All cells that adhered and spread onto coverslips following transfection with constitutively active MKK1 or MKK2 expressed the MKK constructs (F and L).

These results indicate that cells expressing constitutively active forms of MKK lose the capacity to proliferate when they execute a terminal differentiation program.

Activation of ERK upon MKK expression. Constitutively active MKK mutants expressed in fibroblast cell lines lead to the activation of endogeneous ERK (24, 26). In order to verify this effect in K562 cells, MKK and ERK activities were assayed following cell transfection with wild-type or mutant MKK. Expression of wild-type MKK1, wild-type MKK2, or catalytically inactive MKK1 resulted in little elevation of expressed MKK activity (Fig. 3A, lanes 1, 2, and 4) or activation of endogeneous ERK2 (Fig. 3B, lanes 1, 2, and 4). By contrast, expression of constitutively active MKK1 or MKK2 led to significant enhancement of MKK activity (Fig. 3A, lanes 3 and 5) and ERK2 activation (Fig. 3B, lanes 3 and 5), which was augmented by combining both mutants in the transfection (Fig. 3, lanes 6). Activation of ERK2 was further illustrated by retardation of mobility on SDS-PAGE (Fig. 3B), a reflection of its phosphorylation and activation (37). These results demonstrated that K562 cell differentiation induced by constitutively active MKK occurred under conditions that led to stimulation of the MAP kinase cascade.

Effects of MKK expression on platelet-specific markers. Integrin $\alpha_{IIb}\beta_3$ is expressed exclusively on platelets and platelet progenitors, making it a highly specific marker for megakaryocytic differentiation (58). Expression of mRNA for the α_{IIb} and β_3 subunits was examined in K562 cells at various times following PMA treatment or at 96 h posttransfection with MKK. Message levels for both subunits increased significantly in response to PMA (Fig. 4B and D), although the kinetics of α_{IIb} mRNA induction were slower than those of β_3, as previously observed (17, 38, 64). The α_{IIb} message increased 50- to 60-fold and 140-fold in response to expression of constitutively active MKK1 and MKK2, respectively, and 60- to 150-fold upon combining the mutants (Fig. 4A and C). The β_3 message increased 20- to 40-fold and 140-fold in response to constitutively active MKK1 and MKK2, respectively, and 100-fold in the mutant combination. Minimal increases in either message were observed upon expression of wild-type MKK1 or MKK2. Expression of β-actin showed less than threefold variation compared to controls (Fig. 4A and B).

The functional appearance of integrin $\alpha_{IIb}\beta_3$ receptors at the cell surface requires glycosylation and proteolytic cleavage of subunit polypeptides in the endoplasmic reticulum, nonco-

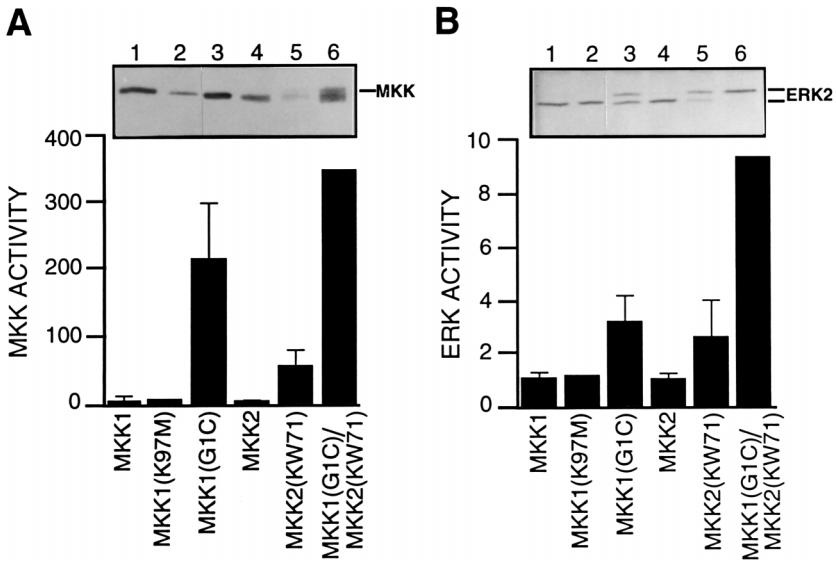

FIG. 3. Activity of expressed MKK and endogeneous ERK2 in K562 cells. Cells were transiently transfected with the indicated HA-tagged MKK constructs and incubated for 48 h before preparation of extracts, immunoprecipitation, and measurement of activity as described in Materials and Methods. (A) Fold activation of HA-MKK activity compared to vector control measured by ^{32}P incorporation into ERK2(K52R). The inset shows the level of immunoprecipitated HA-MKK in Western blots probed with 12CA5 antibody. (B) Fold activation of endogeneous ERK2 activity compared to vector control, measured by ^{32}P incorporation into myelin basic protein. The inset shows the level of immunoprecipitated ERK2 in Western blots probed with anti-ERK2 antibody.

valent association of the transmembrane heterodimer in the Golgi apparatus, and transport to the cell surface (16). To test whether the receptor was processed correctly, the appearance of $\alpha_{IIb}\beta_3$ receptors at the cell surface was probed using a monoclonal antibody which specifically recognizes the intact integrin complex on megakaryocytes (7). PMA treatment caused a 50-fold enhancement of mean fluorescence compared to untreated cells (Fig. 5A and B). Increases of similar magnitude were also observed in cells transiently transfected with constitutively active MKK1, MKK2, or the mutant combination (Fig. 5D, F, and G). In each case, ~25% of the cell population was affected (Fig. 5H), corresponding to the transfection efficiency achieved under these conditions (15 to 25% [data not shown]). This supports the finding made earlier that active MKK mutants induce differentiation only in transfected cells and not through secretion of autocrine differentiation factors. Transfection with wild-type MKK1 or MKK2 led to a modest enhancement of fluorescence, similar to that observed in control cells (Fig. 5C and E). Thus, expression of constitutively active MKK results in the increased expression of functionally intact integrin $\alpha_{IIb}\beta_3$.

PMA stimulation of K562 cells transiently activates MAP kinase. It has been suggested that cell differentiation in PC12 cells requires a prolonged activation of ERK lasting hours to days (39, 51, 60), in contrast to transient activation of ERK occurring in response to proliferative signals. The time course of ERK activation in K562 cells was monitored after cells were treated with a concentration of PMA (50 nM) that induces megakaryocytic differentiation. ERK2 was immunoprecipitated at various times, and activity was measured by phosphorylation of myelin basic protein. ERK2 activity increased rapidly in response to PMA, peaking at 0.5 to 1 h and decreasing after 1 h, finally reaching basal levels by 12 h (Fig. 6). The kinetics of activation measured by immunoprecipitation were paralleled by a shift to a slower-migrating form of ERK2 observed by Western blotting. ERK1, which is 5- to 10-fold less abundant than ERK2 in these cells, responded to PMA with similar kinetics of activation (data not shown). The kinetics of MKK1 and MKK2 activation paralleled those seen with ERK2, and total levels of MKK or ERK did not change with time after PMA treatment (data not shown).

In order to assess whether ERK undergoes nuclear translocation following activation, cells were treated with 50 nM PMA for various times and ERK was visualized by indirect immunofluorescence (Fig. 7). Endogenous ERK was primarily cytoplasmic in untreated cells and in cells treated for 1 h with PMA (Fig. 7A to D); however, localization was primarily nuclear 3 h after PMA treatment (Fig. 7E and F). Most of this staining reappeared in the cytoplasm 6 h after stimulation (Fig. 7G and H) and remained predominantly cytoplasmic up to 24 h (data not shown). These kinetics were confirmed by examining localization of HA-tagged ERK2 expressed in K562 cells (Fig. 8). These results indicate that ERK activation by PMA is followed by nuclear translocation, although nuclear localization appears to occur transiently in this system.

Suppression of K562 differentiation by an inhibitor of MKK. To further test the requirement for MKK/ERK activation during megakaryocytic differentiation induced by PMA, the inhibitor PD98059 was used. This compound has been reported to selectively retard the activation of MKK1 by upstream kinases, thus blocking activation of the MAP kinase pathway in intact cells (15, 34). K562 cells treated with 100 μM PD98059 were viable, and the inhibitor had no measurable effect on cell growth (references 15 and 34 and data not shown). However, the inhibitor caused a significant suppression of endogenous MKK activity and a partial suppression of ERK activation following treatment with PMA (Fig. 9), with a 50% inhibitory concentration for MKK of approximately 5 μM.

The effect of the inhibitor on K562 differentiation was also evaluated. PD98059 had little effect on K562 morphological differentiation in the absence of PMA (Fig. 10A and C) but completely suppressed cell adhesion and spreading normally seen in response to PMA (Fig. 10B and D). In fact, the inhibitor was capable of suppressing the morphological changes when added up to 12 h after PMA stimulation (Fig. 10E and F). In the presence of PD98059 (100 μM), the PMA-induced

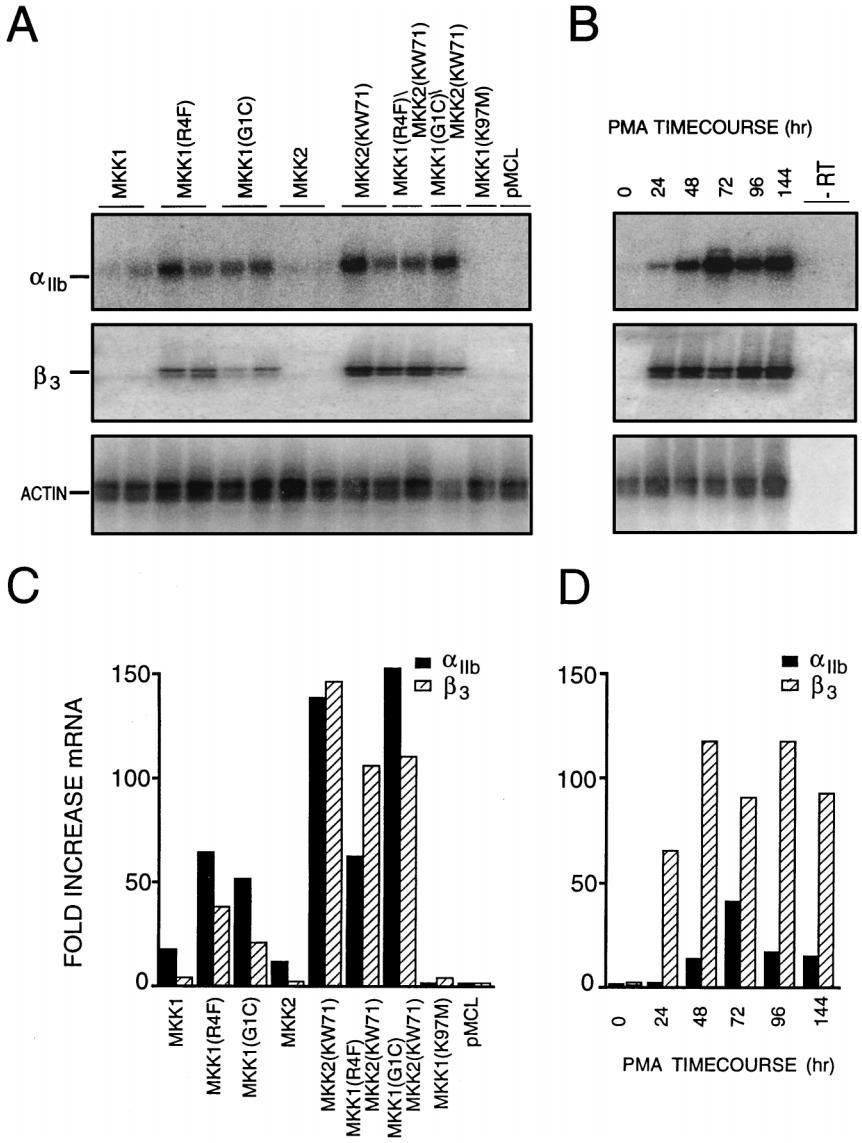

FIG. 4. Message levels of integrin α_{IIb} and β_3 subunits following transfection with MKK. Cells were transiently transfected with the indicated HA-tagged MKK constructs and incubated for 96 h (A and C) or treated with 50 nM PMA for various times before preparation of extracts and isolation of mRNA (B and D). (A and B) Message levels were measured by reverse transcriptase PCR resulting in products from α_{IIb} subunit, β_3 subunit, and β-actin mRNA as indicated in Materials and Methods. −RT, without reverse transcriptase. (C and D) Levels of reverse transcriptase PCR products of α_{IIb} and β_3 subunit messages were quantified by phosphorimager analysis and normalized to cells transfected with empty vector (C) or untreated cells (D). Levels of reverse transcriptase PCR products from β-actin mRNA varied less than threefold compared to controls.

appearance of $\alpha_{IIb}\beta_3$ on the cell surface was reduced to 30% of the level observed after PMA stimulation in the absence of inhibitor (data not shown). PD98059 also reversed the PMA-dependent inhibition of cell growth by 25% (data not shown). The inability to fully suppress integrin $\alpha_{IIb}\beta_3$ cell surface expression or reverse cell growth arrest following PMA treatment most likely reflects incomplete suppression of ERK activation at the highest concentrations of inhibitor. Taken together, these data support a model in which transcriptional induction of megakaryocyte markers and inhibition of cell growth require MKK activation, presumably through ERK activity.

Reversibility of K562 cell differentiation. The ability of PD98059 to suppress cell adhesion and spreading up to 12 h after PMA stimulation (Fig. 10E and F) suggested that certain differentiation responses require elevated MKK activity for prolonged periods. Reversibility of differentiation was examined by removing phorbol ester several hours after treatment. For these experiments, phorbol 12,13-dibutyrate (PDBu), which has lower retention in cell membranes, thus allowing more effective washout, was used. PDBu-induced megakaryocyte differentiation in K562 cells with respect to cell adhesion, cell growth, and integrin expression and kinetics of ERK activation under continuous stimulation were comparable to those observed with PMA (reference 64 and data not shown). Cells were incubated with PDBu, washed, and resuspended in fresh media in the absence of phorbol ester. Washout of PDBu between 1 and 12 h completely inhibited cell adhesion and spreading observed under conditions of continuous treatment (data not shown). Furthermore, washout of PDBu between 1

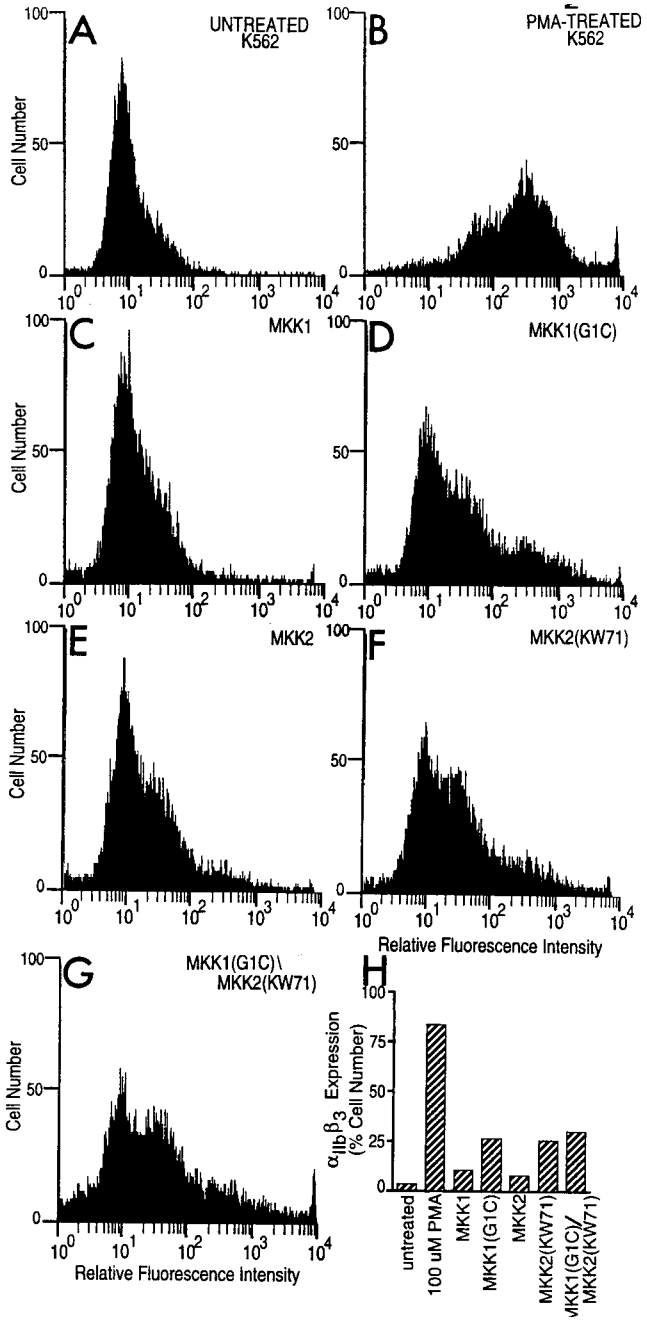

FIG. 5. Flow cytometric analysis of integrin $\alpha_{IIb}\beta_3$ receptors at the plasma membrane. K562 cells were PMA treated or transiently transfected 96 h before staining with 7E3 monoclonal antibody, which recognizes the intact integrin complex, followed by phycoerythrin-conjugated secondary antibody. Fluorescence was quantified as described in Materials and Methods. (A) Untreated cells; (B) cells treated with 50 nM PMA; (C) cells transiently transfected with wild-type MKK1; (D) cells transiently transfected with MKK1-G1C; (E) cells transiently transfected with wild-type MKK2; (F) cells transiently transfected with MKK2-KW71; (G) cells transiently transfected with a combination of MKK1-G1C and MKK2-KW71; (H) bar graph indicating percentage of cells with fluorescence greater than 10^2 U in panels A to G.

and 7 h following treatment led to recovery of cell proliferation, although this recovery was not observed after 24 h of treatment (Fig. 11). These experiments demonstrate the requirement for prolonged signalling several hours beyond the peak of ERK activation measured enzymatically.

Active MKK suppresses erythroid differentiation. K562 cells retain the capacity to differentiate along multiple hematopoietic lineages. For example, erythroid markers, including subunits for fetal ($\alpha_2\gamma_2$) and embryonic ($\varepsilon_2\zeta_2$) hemoglobin, are transcriptionally induced by hemin (8, 41). It has previously been shown that PMA suppresses the transcription of hemoglobin in K562 cells (23). The question of whether MKK, like PMA, not only induces megakaryocytic differentiation but also suppresses alternative cell fates remained.

This was addressed in two ways. First, the effect of the MKK inhibitor on erythroid differentiation was tested with the alpha subunit of hemoglobin (α-globin) as a marker. The basal level of α-globin (Fig. 12A, lane 1) was completely suppressed by addition of either PMA or PDBu (Fig. 12A, lanes 2 and 3). Treatment with hemin increased α-globin levels approximately 10-fold (Fig. 12A, lane 4). PD98059 also elevated basal levels of α-globin (Fig. 12A, lane 5) and blocked the suppression by phorbol ester (Fig. 12A, lanes 6 and 7). However, the inhibitor did not affect α-globin levels induced by hemin (Fig. 12A, lane 8). These results indicate that elevated MKK activity is necessary for suppression of erythroid markers by phorbol esters and further suggest that basal MKK activity maintains the low expression of α-globin observed under unstimulated conditions.

The role of MKK was directly demonstrated by examining the effects of constitutively active MKK transfection on hemin-dependent α-globin expression. Transfection of empty vector, wild-type MKK1, or wild-type MKK2 had little effect on α-globin levels (Fig. 12B, lanes 1, 2, and 6). However, constitutively active MKK1 or MKK2 or the combination of both significantly inhibited hemin-dependent α-globin expression (Fig. 12B, lanes 3, 4, 5, 7, and 8). The same constitutively active MKK mutants also suppressed basal expression of α-globin in untreated cells (data not shown). These results demonstrate that MKK is capable of not only directing megakaryocytic differentiation but also controlling the lineage commitment of K562 cells.

DISCUSSION

In this study, we have demonstrated that K562 erythroleukemia cells differentiate along the megakaryocyte-restricted lineage in response to activation of the MAP kinase pathway and that MKK is necessary and sufficient to cause differentiation. Cells expressing constitutively active MKK1 and/or MKK2 display many hallmarks of normal megakaryocyte development that are induced by phorbol ester treatment, including cell adhesion, increased cell size, and expression of the integrin $\alpha_{IIb}\beta_3$ receptor. The effects are limited to transfected cells and do not involve the induced expression of autocrine differentiation factors. Such behavior of K562 cells resembles the physiological behavior of self-renewing myeloid progenitor cells, in that they must choose between several differentiation programs based on signals received from cytokines (2, 5, 29, 49). Our results indicate that the commitment to the megakaryocyte lineage may be regulated by the MAP kinase pathway in blood progenitor cells. Of particular interest is the finding that the same pathway suppresses expression of hemoglobin, a key marker for erythroid differentiation. To our knowledge, this is the first demonstration of the ability of the MAP kinase pathway to suppress one cell lineage at the same time that it promotes another.

Either constitutively active MKK1 or MKK2 initiated the differentiation process when expressed in K562 cells. The two isoforms have previously been found to differ with respect to autophosphorylation rates, substrate specificity, and recogni-

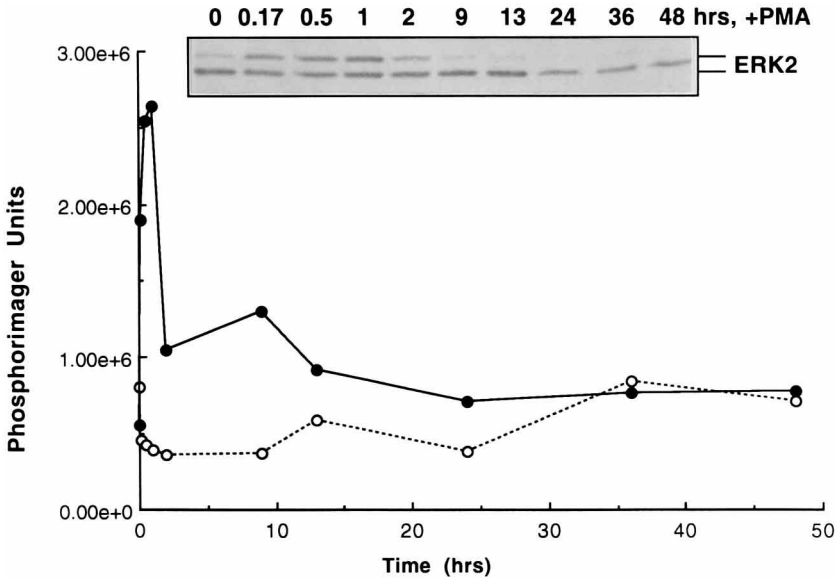

FIG. 6. Activation of ERK in response to PMA treatment of K562 cells. Activity of immunoprecipitated ERK2 was measured 0 to 48 h following treatment of cells with 50 nM PMA (closed circles) or ethanol carrier (open circles) as described in Materials and Methods. (Inset) Western blots of immunoprecipitated ERK2 show retardation in gel mobility correlating with activation.

tion by A-Raf, which might affect their relative activities in vivo (59, 63). However, MKK1 and MKK2 showed little difference in their ability to induce cell transformation or transcriptional activation of TPA response element (TRE)-chloramphenicol acetyltransferase reporter constructs (24, 26). The quantitative differences that we observed between these isoforms, such as in their ability to induce expression of the $\alpha_{IIb}\beta_3$ integrin (Fig. 4A and C), can be accounted for by their relative activities measured in vitro (24, 25). Our results indicate that the two isoforms are equally capable of inducing megakaryocytic differentiation in K562 cells.

Previous studies have provided several examples in which ERK targets nuclear transcription factors for phosphorylation, presumably resulting in the regulation of transcription through various response elements (9, 13, 20, 32, 50, 56). In K562 cells, differentiation induced by PMA or by activation of MKK/ERK causes increased expression of messages for the megakaryocyte-specific integrin $\alpha_{IIb}\beta_3$. These messages have been shown to be upregulated by increased rates of transcription in response to PMA (17, 38, 64). The α_{IIb} gene promoter contains binding sites for GATA and Ets transcription factors (17, 53), and expression of full-length GATA-1 has been shown to induce megakaryocyte differentiation of 416B mouse myeloid cells (54). In addition, a silencer element that suppresses basal expression in the absence of PMA induction has been identified (17). Members of both the GATA and Ets families have been shown to be phosphorylated by ERK (20, 32, 50), suggesting that ERK phosphorylation acts as a transcriptional switch, enhancing the activity of positive transcription factors and/or relieving repression by negative regulators, in conferring lineage-specific differentiation.

Negative regulation of globin genes through transcriptional repression and mRNA destabilization has been demonstrated in K562 cells after PMA stimulation (23); however, the mechanisms for these effects remain obscure. The ability of the constitutively active forms of MKK to suppress α-globin synthesis, combined with the increase in globin expression in the presence of PD98059, indicates that even basal MKK activities repress erythroid differentiation in K562 cells. A role for the

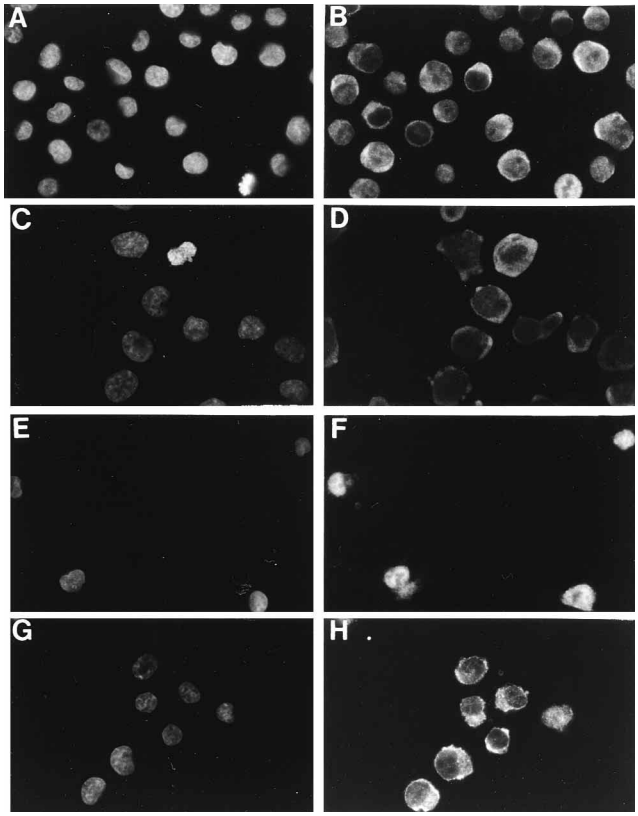

FIG. 7. Nuclear localization of endogenous ERK in response to PMA stimulation. K562 cells were stimulated with 50 nM PMA, fixed, and visualized by DAPI staining of nuclei (A, C, E, and G) and indirect immunofluorescent staining of ERK2 using SC154 antibody (B, D, F, and H). (A and B) Untreated cells; (C and D) cells treated with PMA for 1 h; (E and F) cells treated for 3 h; (G and H) cells treated for 6 h.

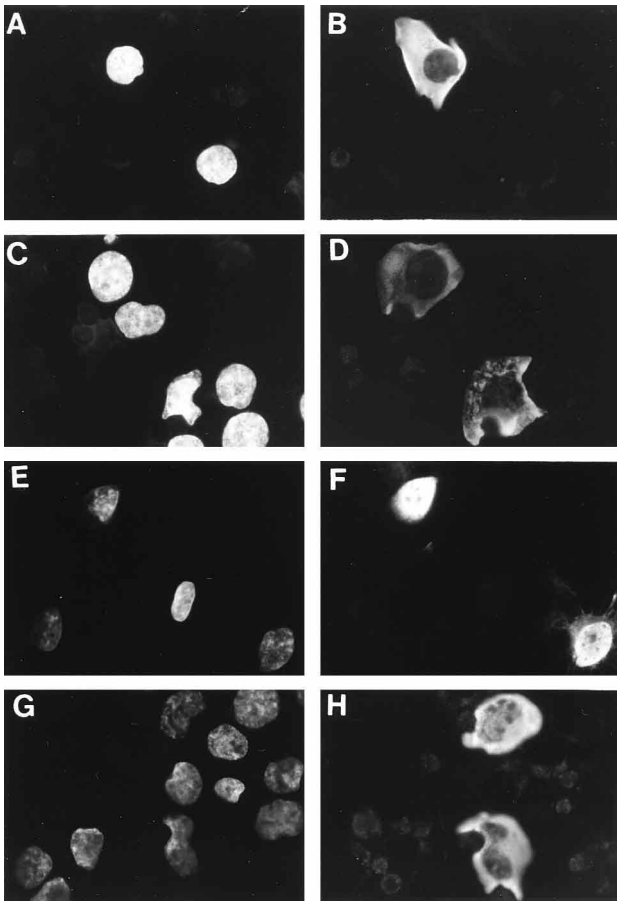

FIG. 8. Nuclear localization of transiently expressed ERK2 in response to PMA stimulation. K562 cells transiently transfected with HA–wild-type ERK2 were stimulated after 24 h with PMA and visualized by DAPI staining of nuclei (A, C, E, and G) and indirect immunofluorescent staining of HA-ERK2 using 12CA5 antibody (B, D, F, and H). (A and B) Untreated cells; (C and D) cells treated with PMA for 1 h; (E and F) cells treated for 3 h; (G and H) cells treated for 6 h.

MAP kinase pathway in transcriptional repression is suggested from studies on the Yan transcription factor in *Drosophila* eye development (32). However, globin transcription is also regulated by locus control region enhancers which govern expression of developmentally regulated globin isoforms in part by regulation of chromatin structure (12, 55). Conceivably, MKK may control a series of events at the level of chromatin organization that represses globin expression.

In PC12 cells, neuronal differentiation correlates with a sustained activation of ERK in response to NGF, overexpression of constitutively active MKK1, or overexpression of epidermal growth factor or insulin receptors followed by factor treatment (11, 27, 39, 51). This prolonged activation can last for up to 10 days and appears to be necessary to maintain the differentiated state and cell survival, since withdrawal of NGF results in apoptosis (60). K562 cell differentiation involves a shorter duration of ERK activation, and the continued presence of the external stimulus is not required for cell survival. In contrast to PC12 cells, removal of phorbol ester from K562 cells appears to reverse effects on both adhesion and cell growth, and inhibition of MKK by PD98059 after the initial activation at least partially blocks further differentiation. Clearly, a transient burst of ERK activity over 1 to 2 h is not sufficient to initiate the megakaryocytic differentiation program, since irreversibility is observed only after 24 h of continuous stimulation. This suggests that submaximal MKK/ERK activities must be experienced by cells over a longer period of time (e.g., one cell cycle) before alterations in transcription, morphology, and cell cycle can be fully achieved.

Studies with PC12 cells also suggest that prolonged activation of ERK leads to nuclear translocation, which has been proposed to explain the correlations between the kinetics of ERK activation and differentiative versus proliferative responses (21, 27). In K562 cells, ERK appears exclusively cytoplasmic at the height of its activity about 1 h after PMA stimulation and is not found in the nucleus until 3 h after activation. Coincident with the transient activity of ERK, nuclear localization is temporary and the protein is found in the cytoplasm 6 h after stimulation. Thus, the timing of nuclear localization provides sufficient opportunity for ERK to phosphorylate nuclear substrates such as transcription factors; however, it does not correlate with the reversal of cell growth or adhesion by MKK inhibitors, indicating that the latter responses may be regulated through separate mechanisms.

Our results also provide evidence for differential thresholds in MKK/ERK activity in influencing different cellular responses. Thus, adhesion and spreading of K562 cells appears to be most sensitive to MKK activation, as these processes are completely blocked by PD98059. Expression of $\alpha_{IIb}\beta_3$ and inhibition of cell growth appears to be less sensitive to the effects of this inhibitor. Evaluation of α-globin expression following MKK transfection also shows differential suppression that correlates with the activity of various MKK mutants, with MKK1-R4F (strongest) showing significant suppression and 11A/55B (weakest) having little effect on either basal or hemin-stimu-

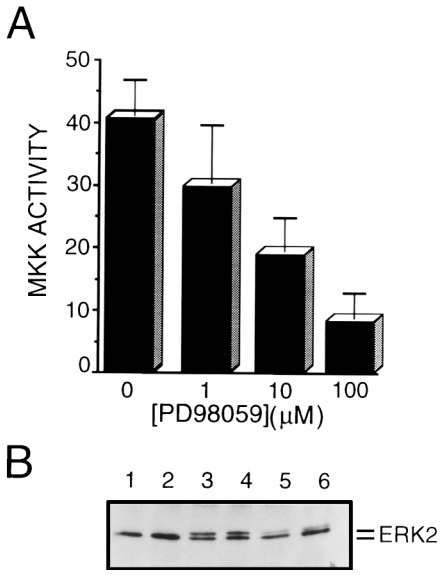

FIG. 9. Inhibition of PMA-stimulated MKK1 and ERK2 activity by PD98059. (A) Activities of endogeneous MKK1 immunoprecipitated with anti-MKK1 antibody were normalized to MKK1 activities in untreated cells. K562 cells were pretreated with various concentrations of PD98059 for 2 h followed by treatment with PMA (50 nM) or ethanol carrier for 45 min. (B) Gel shifts of immunoprecipitated ERK2 in Western blots probed with SC154 antibody. Lane 1, cells treated with ethanol carrier (0.1%, vol/vol) for 45 min; lane 2, cells pretreated with dimethyl sulfoxide (0.1%, vol/vol) for 2 h followed by ethanol for 45 min, lanes 3 to 6, cells pretreated with 0, 1, 10, and 100 μM PD98059, respectively, for 2 h followed by PMA for 45 min.

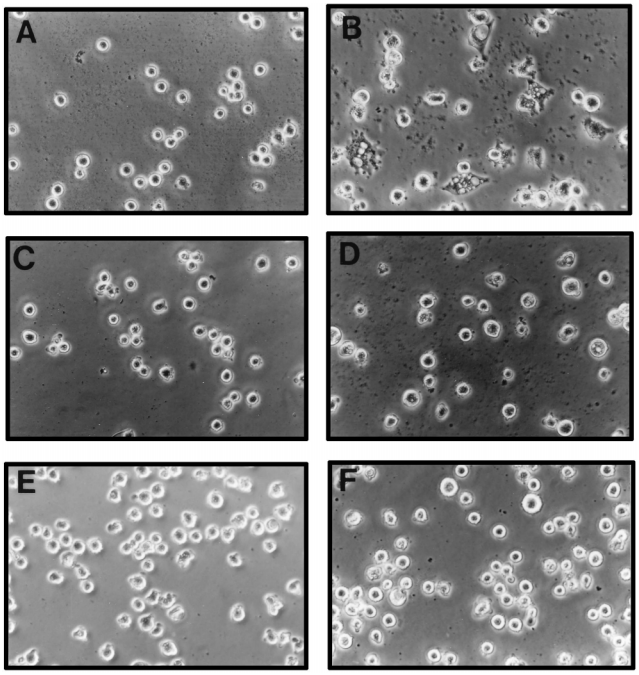

FIG. 10. Inhibition of PMA-induced cell adhesion by PD98059. Cells in petri dishes were visualized by light microscopy. (A) Untreated cells; (B) cells treated with PMA (50 nM) for 48 h; (C) cells treated with PD98059 (50 μM) for 48 h; (D) cells treated with PD98059 for 2 h, followed by PMA addition and continued incubation for 48 h; (E) cells treated with PMA for 2 h, followed by PD98059 addition and continued incubation for 46 h; (F) cells treated with PMA for 10 h, followed by PD98059 addition and continued incubation for 38 h.

lated α-globin expression. However, both constitutively active mutants can induce cell adhesion and spreading.

In our attempts to create stable cell lines, we observed that expression of constitutively active MKK induces cell differentiation and inhibits cell proliferation. This contrasts with the known role of MKK and ERK as positive regulators of cell growth, observed in many cell types (6, 43). We do not know whether the inhibition of cell proliferation reflects an arrest at a specific point in the cell cycle, which is characteristic of differentiation in many cell types. Compounds that induce cell cycle arrest at G_1/S, such as aphidicolin, hydroxylurea, and 1-β-D-arabinofuranosylcytosine, induce erythroid differentiation in K562 cells (14, 29). On the other hand, megakaryocyte differentiation in normal cells initiates an endomitotic process in which cells undergo successive DNA replication events in the absence of intervening mitosis, thus bypassing the requirement for completion of mitosis before entry into S phase (5). Similar effects have been observed in K562 cells (18a). Analysis of other megakaryocytic cell lines indicates that endomitosis may involve upregulation of cyclin D1 (57) and downregulation of cyclin B1/cdc2 kinase activity (62). The inhibition of cell growth that we observe in K562 cells might involve bypass of cell cycle checkpoint control through activation of the MAP kinase pathway.

Several cytokines are known to effect megakaryocyte differentiation in bone marrow cells, including the megakaryocyte colony-stimulating factors interleukin-3 and granulocyte-macrophage colony-stimulating factor, which regulate differentiation of megakaryocyte progenitor cells, and thrombopoetin (TPO), which regulates terminal differentiation (19). These cytokines, alone or in combination with others, govern the commitment of cells along the megakaryocyte pathway, although only TPO has been shown to initiate megakaryocytic

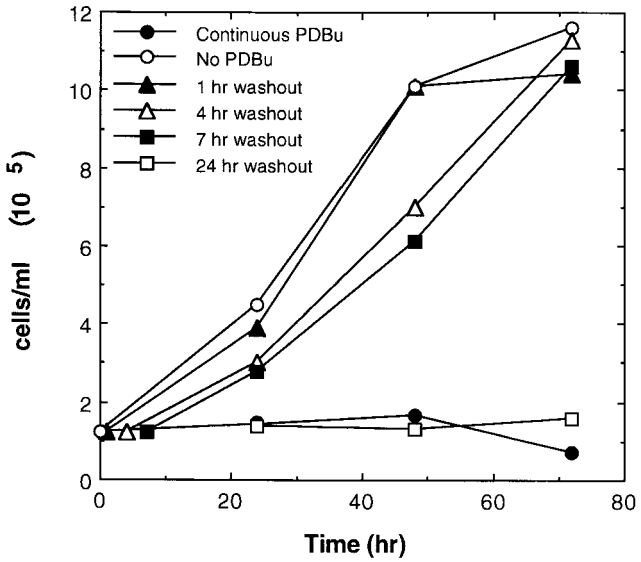

FIG. 11. Reversal of cell growth arrest by phorbol ester. Cells were treated with PDBu (50 nM) or ethanol carrier in a spinner flask. At various times, 5-ml aliquots were washed three times with 10 ml of Hanks' buffered saline solution and seeded into six-well dishes in 5 ml of RPMI medium–10% fetal calf serum without PDBu. Cells were then counted at the indicated times by using trypan blue exclusion to assess viability. Results are expressed as viable cells per milliliter. Continuous PDBu, cells treated for 72 h with PDBu; washout, time of PBDu removal.

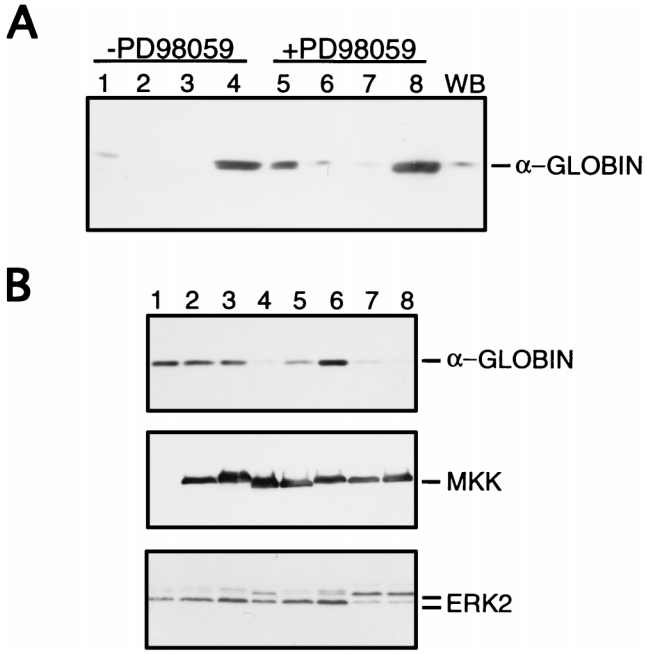

FIG. 12. Effect of MKK on α-globin expression. (A) Western blots of K562 cell extracts probed for α-globin with 3G12 antibody. Cells were incubated for 2 h in the absence or presence of PD98059 as indicated, followed by 48 h with no treatment (lanes 1 and 5) or treatment with 50 nM PMA (lanes 2 and 6), 100 nM PDBu (lanes 3 and 7), or 20 μM hemin (lanes 4 and 8). Lane 8, human α-globin from whole blood extract (WB). (B) Western blots of cells transfected with HA-MKK1 or HA-MKK2 for 24 h followed by addition of hemin and further incubation for 24 h were probed for α-globin, HA-tagged MKK, or ERK as indicated. Transfections were carried out with vector control (lane 1), wild-type MKK1 (lane 2), MKK1-11A/55B (lane 3), MKK1-R4F (lane 4), MKK1-X7, (lane 5), wild-type MKK2 (lane 6), MKK2-KW71 (lane 7), or the combination of MKK1-R4F and MKK2-KW71 (lane 8).

differentiation from pluripotent stem cells in vivo. Because K562 cells lack the TPO receptor in either naive or PMA-treated cells (data not shown), it is difficult to evaluate the effect of this megakaryocyte differentiation factor in this system. However, all of these cytokines have been reported to activate the ERK pathway in other cell types (4, 35, 61), suggesting that the constitutively active MKK mimics cytokine-induced signalling events that regulate normal megakaryocyte differentiation. Our results lay the groundwork to now address the mechanisms by which the MAP kinase pathway regulates cellular differentiation. Current efforts are directed towards understanding how activation of the ERK pathway regulates cell specific gene transcription, inhibits cell cycle progression, and promotes lineage selection in pluripotent cells.

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