The JNK/SAPK Activator Mixed Lineage Kinase 3 (MLK3) Transforms NIH 3T3 Cells in a MEK-dependent Fashion

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ABSTRACT

Mixed lineage kinases (MLKs) form a family of serin/threonine protein kinases with multiple protein/protein interaction domains (SH3, Cdc42 Rac interactive binding sequence, leucine zipper, and proline rich region), the physiological roles of which are largely unknown. We show that overexpression of wild type MLK3 leads to morphological transformation of NIH 3T3 fibroblasts and growth in soft agar. Consistent with this transforming potential, we demonstrate that MLK3 strongly induces transcription from a reporter construct that is driven by a composite AP-1/Ets-1-enhancer element in HEK 293 cells. In the same cell system, MLK3 preferentially activates the c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) mitogen-activated protein kinase cascade and to a lesser degree the extracellular signal-regulated kinase (ERK) pathway. Activation of the latter can be further enhanced by coexpression of wild type MEK1 and is blocked by the synthetic MEK inhibitor PD 098059 or a kinase-dead MEK1 mutant. Immunoprecipitated MLK3 catalyses the phosphorylation of MEK1 in vitro, but this phosphorylation leads only to a marginal activation. In support of these data, we also show that MEK1 is highly phosphorylated in vivo on Ser 217/221 in MLK3-transformed fibroblasts, whereas activating ERK phosphorylations are barely detectable. Nevertheless, MLK3-transformed NIH 3T3 fibroblasts are partially reverted when activation of MEK is specifically blocked with PD 098059. Our combined data show that although MLK3 is primarily an activator of the JNK/SAPK pathway, overexpression of the wild type protein leads to a transformed phenotype in NIH 3T3 cells that can be partially reversed by a synthetic MEK inhibitor. We conclude that the ERK pathway is necessary for MLK3-mediated transformation.

INTRODUCTION

MAPK3 cascades play a key role in converting the signal from extracellular stimuli into intracellular signals regulating differentiation, proliferation, or stress responses (1–4). The best characterized protein kinase cascades are those that activate the ERK-, JNK/SAPK-, and the p38- (also known as RK) activating kinases. In contrast to ERK, JNK/SAPK and p38 are poorly activated by mitogens but strongly activated by inflammatory cytokines, such as interleukin-1β or tumor necrosis factor α. Inducers of cellular stress such as UV, osmotic shock, ionizing radiation, or heat shock also preferentially stimulate JNK/SAPK and p38 (5). Activation of these MAPKs occurs through phosphorylation by a dual-specificity kinase (MAPKK or MKK), which in turn become phosphorylated and activated by ser/thr kinases that are activated at the plasma membrane (6). Activated MAPKs can translocate to the nucleus, where they induce the transcription of specific genes by phosphorylation of transcription factors (1, 7).

Transformation of cells by constitutively active forms of kinases that activate the mitogenic cascade require ERK (8, 9). Expression of a constitutively active form of the ERK-activating kinase MEK1 elicits transformation (8, 10, 11). So far, only the activation of the classic mitogenic cascade (Raf-MEK-ERK) has been convincingly implicated in oncogenesis. However, recent evidence supports a functional role for JNK/SAPK in transformation. A previously published partial clone of JIP-1, which specifically binds JNK/SAPK, thereby preventing its activated form from shuttling into the nucleus, inhibits transformation of pre-B cells by the leukemogenic oncopogene bcr-abl (12). Additionally, transformation of NIH 3T3 fibroblasts by the v-Crk adapter protein is blocked by a dominant negative mutant of the JNK/SAPK-activating kinase SEK1/MKK4 (13). The transcription factor complex AP-1, a major downstream target of MAPK cascades, is a heterodimer made up by Fos, Jun, and ATF basic region ZIPs family proteins (14, 15). Activated JNK/SAPK phosphorylates c-Jun on Ser residues 63 and 73 within the transactivation domain, which increases the half-life of c-Jun, thereby leading to an accumulation of the protein as well as enhanced trans-activation and DNA-binding activity (14). Overexpression of c-Jun transforms chicken embryo fibroblasts (16), and Jun is required for transformation of fibroblasts by a variety of oncogenes (17–20).

The members of the MLK family (MLK1, MLK2, and MLK3) all contain an SH3 domain, followed by the kinase domain that shows highest amino acid identity with TAK and c-Raf1 (21). Additional interaction domains include a potential binding site for Cdc42 and Rac (22) and ZIP domains. MLKs are highly homologous in their SH3, kinase, and ZIP domains (23), suggesting a conserved function, whereas they differ in their COOH-terminal regions. Recently, it has been reported that MLK3, also referred to as SPRK or PTK1 (24–26), is a direct activator of the dual specificity kinase SEK1/MKK4 (27–29).

In this study, we demonstrate that overexpression of wild type MLK3 results in the transformation of NIH 3T3 fibroblasts and anchorage-independent growth. Transient overexpression of MLK3 in HEK 293 cells leads to a marked increase in JNK/SAPK activity, which is also observed in stably MLK3-transformed NIH 3T3 cells. In support of these findings, we also observe that in these cells c-Jun is constitutively phosphorylated in vivo. In addition, overexpression of MLK3 in HEK 293 cells results in a moderate activation of the MEK1-ERK protein kinase pathway, which can be blocked by the synthetic MEK inhibitor PD 098059 or a KD MEK1 mutant. Further experiments show that MEK1 is a potent target of MLK3 in vitro and in vivo, but this MEK1 phosphorylation merely results in a subtle ERK activation. Although constitutive activation of ERK is not detectable in MLK3-transformed fibroblasts, the MEK inhibitor PD 098059 partially reverts the transformed phenotype, indicating that the mitogenic cascade is required for the full MLK3-transformed phenotype.

MATERIALS AND METHODS

Cell Lines and Antibodies. The human embryonic kidney cell line HEK 293 and mouse NIH 3T3 fibroblasts were cultured in DMEM supplemented with 10% FCS (heat-inactivated at 56°C for 45 min), 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin at 37°C in humidified air with 5% CO2. Soft agar cloning was performed, as described before (30). The following primary antibodies were used for the indicated antigens: Flag-tag, D-8 (rabbit

Received 2/5/99; accepted 3/4/99.

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1 Supported by a grant from the Deutsche Forschungsgemeinschaft (Sonderforschungs- bereich 172).

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3 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; SAPK, stress-activated protein kinase; MLK, mixed lineage kinase; MEK, MAPK/ERK kinase; SEK, SAPK/ERK kinase; MKK, MAPK kinase; ZIP, leucine zipper; RK, reactivating kinase; KD, kinase-dead.
polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA; MLK3, C-20 (rabbit polyclonal; Santa Cruz Biotechnology); Flag-tag, M2 (mouse monoclonal; Eastman Kodak); phospho-ERK1/2, E-4 (mouse monoclonal; Santa Cruz Biotechnology); ERK1, C-16 (rabbit polyclonal; Santa Cruz Biotechnology); phospho-MEK, (mouse monoclonal; NEB); MEK1, C-18 (rabbit polyclonal; Santa Cruz Biotechnology); c-Jun/AP-1, N (rabbit polyclonal; Santa Cruz Biotechnology); phospho-c-Jun, KM-1 (mouse monoclonal; Santa Cruz Biotechnology); cdk4, C-22 (rabbit polyclonal; Santa Cruz Biotechnology); HA-tag, 12CA5 (mouse monoclonal; Babco).

**DNA Constructs.** The cDNAs encoding NH₂-terminally HA (influenza haemagglutinin)-tagged rat p44ERK1 and rat p54SAPKβ (provided by Dr. Jim Woodgett, Ontario Cancer Institute, Toronto, Canada) were inserted into the mammalian expression vectors pRSPA or pMT2, respectively (31, 32). The cDNAs for c-Raf1, Raf-C4B, Raf-BXB (33), as well as MLK3 (provided by Drs. K. Gallo and P. Godowski, Genentech, San Francisco, CA), were subcloned into pRSPA (31). Raf-C4B is a dominant-negative form of c-Raf1, whereas Raf-BXB is constitutively active. MLK3 cDNAs (MLK3 wild type and MLK3 K144A) were also subcloned into the SalI sites of the retroviral vector pBabe puro (34) and the EcoRI-BamHI sites of the mammalian expression vector pCMV5. In the MLK3 K144A mutant, the kinase activity is abolished by exchange of the ATP binding site lysine to alanine. Construction of a haemagglutinin-tagged version of Raf-BXB has been described previously (35). Wild type MEK1 coding sequences were released from the pBabe puro vector (provided by S. Cowley, Chester Beatty Research.

Fig. 1. Overexpression of MLK3 wild type is sufficient to transform NIH 3T3 fibroblasts. NIH 3T3 cells were transfected with an empty vector (pBabe puro) or expression constructs for KD (MLK3 K144A) or wild type MLK3 and selected for puromycin resistance. After 24 days, the cells were fixed with methanol and stained with giemsa.

Fig. 2. Morphology of MLK3-transformed NIH 3T3 fibroblasts. Morphology of NIH 3T3 cells transfected with a vector control or expression vectors for KD (MLK3 K144A) or wild type MLK3 (A–C). MLK3-transformed fibroblasts are refractile and spindle-shaped (C). Pictures were taken 24 days after transfections. Cells infected with MLK3 wild type form colonies in soft agar (D).
Laboratory, London, England) after cleavage with BamHI and EcoRI and ligated into the BamHI-EcoRI sites of pCDNA3. pR5K-MEK1 K97M was generated by inserting a BamHI-HindIII fragment from pSESTA-MEK1 K97M (provided by N. Ahn, University of Colorado, Boulder, CO) into pR5K. Construction of a retroviral v-Raf (EHi neo expression construct) was described before (36). The pB4x luciferase reporter construct has been generated by replacement of the CAT (33) by the luciferase gene.

**Transfections, Reporter Gene Assays, and Kinase Assays.** NIH 3T3 fibroblasts were seeded at 1 x 10^5 cells/well of a 6-well plate and grown for 24 h before transfection using high-efficiency liposome transfection method (lipofectamine; Life Technologies, Inc.), as described previously (37). HEK 293 cells were seeded at 5 x 10^5 cells/well of a 6-well plate and grown for 24 h before transfection. Up to 4 μg of plasmid DNA were transfected using a modified calcium phosphate coprecipitation method (38). DNA content was normalized with appropriate empty expression vectors. Cells were starved for 36–48 hours in DMEM containing 0.3% FCS before stimulation.

Stimulation was carried out with 0.5 mM sodium-meta-arsenite (Sigma Chemical Co.) for 40 min or 10% FCS for 5 min. The MEK-specific inhibitor PD 098059 (Calbiochem) was used at a final concentration of 30 μM. Chemokine Co.) for 40 min or 10% FCS for 5 min. The MEK-specific inhibitor PD 098059 (Calbiochem) was used at a final concentration of 30 μM. Cell lysates were prepared as described previously (37). Immunoblot analysis was carried out as described previously (37).

**RESULTS**

**NIH 3T3 Fibroblasts Overexpressing MLK3 Wild Type are Morphologically Transformed and Form Colonies in Soft Agar.** To test for the transforming potential of MLK3, both the wild type and a KD version (MLK3 K144A) of the protein were cloned into the pBabe puro retrovector, and the resulting plasmids were transfected into NIH 3T3 cells. The empty vector was used as a negative control. Twelve to 15 days after puromycin-resistant fibroblasts had reached confluency, isolated foci consisting of 100–250 cells became visible in the MLK3 wild type-transfected population (Fig. 1). The cell morphology was similar to v-Raf-transformed NIH 3T3 cells (Fig. 2 and data not shown). Fibroblasts transfected with an empty vector or KD MLK3 did not form foci within 30 days (Fig. 1). Expression of the Flag-tagged MLK3 proteins in these cells was confirmed by Western blot analysis using an anti-Flag antibody (data not shown). Colony formation in soft agar was assayed to study anchorage-independent growth. Colonies of NIH 3T3 cells infected with viruses expressing the MLK3 protein were easily detected after 15 days (Fig. 2). No colonies in soft agar were observed with an empty vector (data not shown).

**MLK3 Wild Type Activates Transcription from an AP-1-/Ets-driven Promotor.** A variety of oncogenes have been shown to up-regulate transcription from promoters containing AP-1-/Ets-binding sites (33, 42). We, thus, analyzed the ability of MLK3 to activate transcription from the pB4x-luciferase reporter construct, which contains four copies of an oncogene-responsive element derived from the polyomavirus enhancer inserted upstream of the β-globin promoter fused to the luciferase gene (42). Each of the four copies contains an AP-1- and an overlapping Ets-1-binding site. In HEK 293 cells, wild type MLK3 led to a 25-fold induction of the reporter gene compared with the vector control. Expression of activated c-Raf1 (Raf-BXB), which was used as a positive control, resulted in a 36-fold induction. Furthermore, a KD mutant of MLK3 (MLK3 K144A) blocked basal transcription by about 50% (Fig. 3). This clearly demonstrates that the kinase domain of MLK3 is able to activate transcription from the oncogene response element in the pB4x-luciferase reporter construct.

**Overexpression of MLK3 Leads to Activation of JNK/SAPK as well as ERK MAPK.** AP-1 and Ets transcription factors are well-defined targets of MAPK signal transduction cascades (43, 44). To

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*Fig. 3. MLK3 strongly induces AP-1-/Ets-1-dependent transcription in HEK 293 cells. HEK 293 cells were transiently transfected with 0.5 μg of pB4X luciferase reporter construct plus 0.5 μg of RSV-β-galactosidase expression vector together with 2 μg of MLK3 wild type, MLK3 K144A (KD), Raf-BXB, or the corresponding empty expression vector pBSPA. Thirty-six hours after transfections, cells were harvested, and luciferase activity was measured. The results were normalized on the basis of β-galactosidase activity and the vector control was arbitrarily chosen as 1. Mean values and SD obtained from three independent experiments, each done in triplicate, are shown.*
determine whether MLK3 can activate MAPKs, MLK3 was transfected into HEK 293 cells together with either epitope-tagged ERK1 or SAPKβ. The MAPKs were immunoprecipitated, and their activities measured in an in vitro kinase assay. As shown in Fig. 4A, expression of MLK3 strongly activated SAPKβ and does not affect arsenite-mediated SAPK activation. Expression plasmid (1 μg) for HA-SAPKβ was cotransfected with 2 μg of the indicated plasmids, and SAPK activity was measured in an in vitro kinase assay with GST-c-Jun as a substrate. Arsenite stimulation was performed at a concentration of 0.5 mM 40 min before cell lysis. B. MLK3 activates ERK1 and does not affect ERK1 activation by serum growth factors (left). MLK3 activates ERK1 independent of Raf (right). Expression plasmid (1 μg) for HA-ERK1 was cotransfected together with 2 μg of the indicated plasmids, and ERK activity was measured with myelin basic protein as a substrate. Cells were stimulated with serum at a final concentration of 10% 5 min before cell lysis. C. MLK3 activation of ERK is sensitive to the synthetic MEK inhibitor PD 098059 (left). MLK3 synergizes with MEK wild type in the induction of ERK1, whereas KD MEK1 (K97M) abolishes MLK3-mediated ERK1 activation (right). Kinase assays were performed as described above. The synthetic MEK inhibitor PD 098059 was used at a concentration of 30 μM. Preincubation with the inhibitor or the solvent DMSO was started immediately after transfection. The expression of the MAPK constructs was controlled by immunoblotting.

MLK3 Is a Weak Activator of MEK1 in Vitro. Activation of SEK1 by MLK3 results from direct phosphorylation (27–29). To test whether MLK3 is also able to directly phosphorylate and activate ERK stimulation is independent of Raf and Ras (Fig. 4B). To further investigate the pathway by which MLK3 activates ERK in 293 cells, a specific inhibitor of MEK, PD 098059 (46), was used. As shown in Fig. 4C, this drug efficiently blocked ERK activation by MLK3 and serum, whereas under the conditions used it only partially blocked Raf-BXB-induced ERK activation. Furthermore, coexpression of wild type MEK1 with MLK3 resulted in a strong increase in ERK activity (Fig. 4C). Requirement for MEK1 in MLK3-induced ERK activation is further supported by the finding that a KD mutant of MEK1 (MEK1 K97M) almost completely abolished MLK3 as well as Raf-induced ERK activation (Fig. 4C). Thus, overexpression of MLK3 results in a pronounced stimulation of JNK/SAPK and, to a lesser degree, also of ERK. ERK activation is independent of Raf, but sensitive to a synthetic MEK inhibitor and kinase-dead MEK1.
MEK1 epitope-tagged versions of MLK3 or Raf-BXB were transiently expressed in HEK 293 cells, isolated from cell extracts by immunoprecipitation and washed extensively. Afterward, the kinases were incubated with purified recombinant KD His-MEK1 or GST-SEK1, respectively, in the presence of [γ-32P]ATP. Wild type, but not KD, MLK3 was able to phosphorylate His-MEK1 (Fig. 5A) and GST-SEK1 (Fig. 5B) in vitro. Using a coupled assay, we were able to show that MLK3-mediated phosphorylation of SEK1 resulted in activation because GST-SEK1 is able to phosphorylate recombinant SAPKα (Fig. 5D). The ability of immobilized MLK3 to activate wild type GST-MEK1 was assayed with KD His-ERK1 as a substrate. Though both MLK3 and the known MEK activator c-Raf1 phosphorylate MEK1 (Fig. 5, A and C), the degree of activation measured by phosphorylation of His-ERK1 K52R was much lower in the case of MLK3 compared with Raf-BXB (Fig. 5C).

**MEK1 and c-Jun Are Constitutively Phosphorylated in MLK3-transformed NIH 3T3 Fibroblasts.** To study the status of MAPK pathways in NIH 3T3 cells expressing exogenous MLK3 proteins, we compared different cell lines with v-Raf-transformed cells. The flag-tagged MLK3 transgene is significantly overexpressed compared with endogenous MLK3 in all transformants analyzed (Fig. 6). It has been reported in the past that JNK/SAPKs phosphorylate the transcription factor c-Jun more efficiently than ERKs (47, 48), whereas stimulation of c-Jun synthesis is triggered by ERKs alone (19, 49). Serum-starved cells express low levels c-Jun (Fig. 6, Lane 1), which shifts to slower mobility after arsenite stimulation (Fig. 6, Lane 7). In MLK3 overexpressing fibroblasts, c-Jun was shifted and its expression was increased, whereas v-Raf essentially only increased the expression. To confirm that the shifted c-Jun is, indeed, phosphorylated, we used a c-Jun antibody that preferentially recognizes phosphorylated c-Jun. In support of our data, MLK3-transformed cell lines (Fig. 6, Lanes 4 and 5) show a high degree of phosphorylated c-Jun, which is most likely due to the presence of constitutively activated JNK/SAPKs in these cells (data not shown).

To test for MEK-ERK activity, antibodies that recognize the phosphorylation of critical residues required for activation, were used. Our data show that although MEK1 is strongly phosphorylated, in vivo ERK phosphorylation is not up regulated in MLK3 expressors (Fig. 6, Lanes 4 and 5), whereas both are activated in v-Raf-transformed cells (Fig. 6, Lane 6). However, in transient experiments in NIH 3T3 cells, coexpression of MLK3, together with epitope-tagged ERK, results in activation of ERK, demonstrating that MLK3 is capable of activating ERK in this cell system (data not shown). In a MLK3-expressing cell line generated by selection in puromycin, which only shows low levels of transgene expression (Fig. 6, Lane 3), hardly any phosphorylation of MEK or c-Jun could be detected. This is also consistent with a low number of transformed cells in this population. In cells
isolated by soft agar cloning, we detect much higher amounts of exogenous MLK3 (Fig. 6, Lanes 4 and 5), suggesting that anchorage-independent growth and transformation require high levels of MLK3 expression.

The MEK Inhibitor PD 098059 Partially Reverts the MLK3-transformed Phenotype. To analyze the role of the Raf-MEK-ERK cascade in MLK3-mediated transformation, we studied the effect of a specific inhibitor of this pathway. MLK3-transformed cells are spindle-shaped and refractile, but flatten out in the presence of the MEK inhibitor PD 098059 (Fig. 7A). Cells transformed with v-Raf were chosen as a positive control and showed an even stronger reversion. The same results were obtained with UO126, a novel MEK-specific inhibitor that has been described recently (data not shown; Ref. 50). In MLK3- and Raf-transformed cell lines incubated with the inhibitor PD 098059 (Fig. 7B, Lanes 2 and 4), ERK1/2 phosphorylation is markedly decreased, whereas ERK1/2 protein expression is not effected, suggesting that activation of ERK1/2 is necessary to achieve the fully transformed phenotype.

DISCUSSION

In this study, we demonstrate that overexpression of the serine/threonine protein kinase MLK3 in NIH 3T3 fibroblasts results in transformation and anchorage-independent growth. Consistent with the transforming activity, wild type MLK3 strongly induces transcription from a reporter construct that is driven by a composite AP-1/-Ets-1-(PEA3)-enhancer element, which was shown previously to be responsive to serum, 12-O-tetradecanoylphorbol-13-acetate, and diverse classes of oncogenes (33, 42). Because both AP-1 and Ets-1 transcription factors are targets for different MAPK cascades, we analyzed the role of MLK3 in the activation of these pathways. Our data demonstrate that MLK3 is primarily an activator of JNK/SAPKs and, to a lesser degree, of ERKs. In addition, MEK activation is required for MLK3 transformation of NIH 3T3 cells.

Of the oncogene class of transcription factors that are regulated through MAPK cascades, p62^{TCF} (elk-1, sap-1; Ref. 43 and 44) belongs to the Ets family, whereas Fos and Jun family members form the AP-1 complex (14, 15). In this study, we have used a reporter construct that consists of four copies of a composite AP-1/-Ets-1-(PEA3)-enhancer element that was initially described as the Ras-responsive element in the poliovirus enhancer (42). Similar sites were shortly afterward identified in the promotor of a number of cellular genes (44) and shown to respond to diverse classes of oncogenes (33, 42, 51). We demonstrate that wild type MLK3 strongly induces transcription from this reporter construct comparable with activated Raf, consistent with the transforming potential of the kinase. In agreement with earlier reports, dominant-negative versions of Raf strongly reduce basal transcription from this AP-1/-Ets-1-enhancer element (33). In this study, we demonstrate the same effect for kinase-dead MLK3 (Fig. 3), further underlining the importance of MLK3 in the regulation of this enhancer element.

It has been shown recently that MLK3 can activate the JNK/SAPK MAP kinase (27–29), but does not affect ERK MAP kinase activity. We provide evidence that although MLK3 primarily activates the JNK/SAPK signaling pathway by demonstrating that: (a), overexpression of MLK3 leads to the activation of ERK; (b), this effect can be significantly increased by cotransfection of MEK1 wild type; (c), MLK3-mediated activation of the ERK pathway can be completely blocked by a synthetic MEK inhibitor or with kinase-dead MEK1; (d), MLK3 phosphorylates bacterially expressed KD MEK1; and (e), MLK3 modestly activates wild type MEK1 in a coupled kinase assay using KD ERK2 as substrate. The reason for the discrepancy observed between efficient MEK phosphorylation and failure to substantially activate MEK in a coupled assay is currently unknown. It has been reported in the past that the previously identified MEK activators Raf, Mos, and TPL2/COT activate MEK through phosphorylation of Ser 217/221 (10, 52, 53). MEK kinase 1 (MEKK1), first identified as a MEK activator (54), was shown later to preferentially activate SEK1/MKK4-JNK/SAPK (55). MEKK1-mediated activation of MEK1 also results from phosphorylation of these two serine residues, but only leads to minimal ERK activation (56). Because MLK3 phosphorylation of bacterially expressed MEK1 also does not lead to full activation of the kinase when compared with Raf activation, we cannot exclude that MLK3 might use additional phosphorylation sites other than Ser 217/221 or that additional factors (e.g., phosphatases, interaction with scaffold proteins) govern the outcome of MEK1 phosphorylation.

Many oncoproteins have been shown to require activation of the
Raf-MEK-ERK cascade for transformation (8, 9, 10, 46). In MLK3-transformed NIH 3T3 cells, we demonstrate that although MEK is strongly activated, ERK phosphorylation as detected by phosphorylation site-specific antibodies is not affected. However, this does not exclude a requirement for the ERK kinase in MLK3-mediated transformation. Similar to our data, it was reported recently that in v-Src or v-Ha-Ras-transformed Rat fibroblasts, ERK1/2 activity was repressed although MEK1/2 was constitutively phosphorylated (57). The latter report presented evidence that an unidentified single-specificity tyrosine phosphatase is responsible for ERK1/2 inactivation. A similar situation might exist in MLK3-transformed cells. In fact, as we show here, transiently expressed MLK3 is capable to activate ERKs in NIH 3T3 cells and, more convincingly, MLK3-transformed NIH 3T3 cell lines incubated with the MEK inhibitor PD 098059 showed partial reversion of their transformed morphology (Fig. 7A).
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