Induction of Mitogen-activated Protein Kinase Phosphatase 1 by the Stress-activated Protein Kinase Signaling Pathway but Not by Extracellular Signal-regulated Kinase in Fibroblasts*

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The intracellular mechanisms involved in the activation of extracellular signal-regulated kinase (ERK) are relatively well understood. However, the intracellular signaling pathways which regulate the termination of ERK activity remain to be elucidated. Mitogen-activated protein kinase phosphatase 1 (MKP-1) has been shown to dephosphorylate and inactivate ERK in vitro and in vivo. In the present study, we show in NIH3T3 fibroblasts that activation of the stress-activated protein kinase (SAPK) pathway by either specific extracellular stress stimuli or via induction of MEKK, an upstream kinase of SAPK, results in MKP-1 gene expression. In contrast, selective stimulation of the ERK pathway by 12-O-tetradecanoylphorbol-13-acetate or following expression of constitutively active MEK, the upstream dual specificity kinase of ERK did not induce the transcription of MKP-1. Hence, these findings demonstrate the existence of cross-talk between the ERK and SAPK signaling cascades since activation of SAPK induced the expression of MKP-1 that can inactivate ERK. This mechanism may modulate the cellular response to stimuli which employ the SAPK signal transduction pathway.

Mitogen-activated protein (MAP)1 kinases are important components in the intracellular regulatory network that transduce extracellular cues to intracellular responses. The complete reconstitution of two distinct Ras-dependent MAP kinase cascades has been described in mammalian cells. One is the intensively investigated Raf-MEK-ERK cascade and the other recently described kinase cascade is initiated by MEKK (MEK kinase) leading to activation of SEK1, the upstream dual specificity kinase of stress-activated protein kinase (SAPK), that in turn phosphorylates and activates SAPK. Extracellular signal-regulated kinase (ERK) mediates cellular proliferation and differentiation (1) whereas the SAPK, also referred to as Jun-N-terminal kinase (JNK), pathway plays a pivotal role in the response to extra- and intracellular stress stimuli and generally promotes inhibition of cell growth (2–4).

The expression of MKP-1 is principally regulated at the transcriptional level (8, 9), we have studied the MKP-1 gene expression in response to induction of both ERK or SAPK in order to determine the signaling cascades involved in the regulation of MKP-1. We demonstrate the induction of MKP-1 in response to activation of the SAPK signaling pathway but not after stimulation of ERK. Since MKP-1 is capable of inactivating ERK, its induction may play a pivotal role in the cellular stress response.

Experimental Procedures

Materials—The ECL system was obtained from Amersham, Dulbecco’s modified Eagle’s medium from Life Technologies, Inc., and [32P]dCTP from DuPont NEN. TPA, anisomycin, and all other reagents were obtained from Sigma.

Cell Culture—NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO2, 95% air mixture. Cells were made quiescent for 24 h in serum-free media. Prior to stimulation with UV-light, the medium was removed and saved. Cells were washed once with phosphate-buffered saline, stimulated, and reincubated in the original medium for different periods of time.

Expression of Mutants of MEK and MEKK—As described previously (10), NIH3T3 cells were stably transfected with pBS2neo vector (Clontech) alone or together with pMM9 vectors encoding wild-type (WT) MEK, catalytically inactive MEK, or constitutively active MEK under control of the Moloney sarcoma virus long terminal repeat (10). The constitutively active mutant of MEK (ΔN3-5222) was designed by substitution of a regulatory site, Ser222, with aspartic acid and deletion of a predicted α helix encompassing residues 32 to 51 and a catalytically inactive mutant (K97M) by substitution of Lys97 with methionine in the ATP binding site (10).

As described previously (3), the EE epitope-tagged C-terminal 320 amino acids of MEKK1 (ΔMEKK) was expressed in NIH3T3 cells using the lacSwitch promoter (Stratagene). Parental cells and stably transfected cells that express catalytically active ΔMEKK in response to isopropyl-1-thio-β-D-galactosidase (IPTG) were incubated with 1 mM IPTG for the indicated times.

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RNA Extraction and Northern Blot Analysis—Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform procedure (11). The quality of RNA was assessed by measuring the ratio of 28S:18S rRNA (2:1) in ethidium-stained denaturing agarose gels. For Northern analysis, RNA was denatured by heating for 15 min at 65°C in 0.02 M MOPS, 6.6% formaldehyde, and 50% formamide and fractionated by electrophoresis in a 1.2% agarose gel. The RNA was then transferred to nitrocellulose. MKP-1 mRNA was detected by hybridization of the cell cultures with CL100 cDNA (12) (generous gift of S. M. Keyse, Dundee, United Kingdom), the human homologue of MKP-1 (97% identity) (6), labeled bynick translation with [32P]dCTP. The hybridization buffer contained 50% formamide, 5 × SSPE, 0.5% SDS, 10% dextran sulfate, 1 × Denhardt’s, and 110 μg/ml salmon testes DNA. After hybridization overnight, the membranes were washed twice with 2 × SSPE, 0.1% SDS at 20°C for 10 min and twice with 0.1 × SSPE, 0.1% SDS at 42°C for 20 min for CL100. After stripping, each blot was rehybridized with glyceraldehyde-3-phosphate dehydrogenase cDNA probe. The amount of mRNA expression was quantified by densitometry using National Institutes of Health IMAGE software on an Apple Macintosh computer equipped with a Microtech scanner and corrected for glyceraldehyde-3-phosphate dehydrogenase gene expression. Autoradiograms are from representative experiments.

Western Blot Analysis—Confluent NIH3T3 fibroblasts were lysed in 400 μl of Triton X-100 lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 5 mM EDTA, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml phenylmethylsulfon fluoride, 200 μm sodium orthovanadate) at 4°C. After 5 min, cell lysates were centrifuged at 4°C for 15 min at 10,000 × g. The soluble cell lysates were mixed 1:4 with 5 × Laemmli buffer and heated for 5 min at 95°C. Soluble cell lysates (80 μg) were loaded per lane and separated by SDS-PAGE using 4% and 10% acrylamide for stacking and resolving gels, respectively. Protein was transferred to nitrocellulose and probed with polyclonal antibodies raised against the C-terminal peptide of either p42ERK (13) or p46SAPK (14). The primary antibodies were detected using horseradish peroxidase-conjugated protein A visualized by Amersham ECL system after intensive washing of the membranes.

ERK Activity Assay—400 μg of soluble cell lysates (as described above) were incubated with 90 min with 2 μl of polyclonal antibody recognizing p42ERK (13). Immunocomplexes were adsorbed to protein A-Sepharose, washed twice with lysis buffer and twice with kinase buffer (10 mM MgCl2, 20 mM HEPES, pH 7.4, containing 200 μM sodium orthovanadate), and resuspended in 60 μl of kinase buffer containing 0.25 mg/ml myelin basic protein, 50 μM ATP, and 5 μCi of [γ-32P]ATP. The reaction was initiated by incubation at 30°C for 15 min. Thereafter, 60 μl of 2 × Laemmli buffer was added to terminate the reaction, and samples were subjected to SDS-PAGE (12%) gel.

RESULTS AND DISCUSSION

After stimulation of quiescent, parental NIH3T3 cells with serum, MKP-1 expression was induced rapidly (Fig. 1A). To investigate the regulation of MKP-1 gene expression, we used extracellular stimuli that activate either the ERK or the SAPK short wavelength UV-light (UV-C) on the activation of ERK and SAPK in NIH3T3 cells. A, quiescent NIH3T3 cells were stimulated with 10% fetal bovine serum (FBS), TPA (100 nm), UV-C (40 J/m2), or anisomycin (500 nm) for different time periods as indicated. Total cellular RNA was isolated as described above. MKP-1 mRNA (2.2 kilobases in length) was detected by Northern blot analysis. Blots were reprobed with glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) cDNA from NIH3T3 cells as a loading control. B, quiescent NIH3T3 cells were incubated with TPA (100 nm), anisomycin (500 nm), or UV-C (40 J/m2) for 5 and 30 min. Both ERK isoforms, p42 and p44, are detected in whole cell lysate by Western blot. Activation is identifiable by the appearance of bands with delayed mobility indicating phosphorylated protein forms (indicated by stars). C, quiescent NIH3T3 cells were stimulated with TPA (100 nm), anisomycin (500 nm), or UV-C (40 J/m2) for 5 and 30 min. Both SAPK isoforms, p46 and p54, are detected in whole cell lysate by Western blot as described above. Activation is identifiable by the appearance of bands with delayed mobility indicating phosphorylated protein forms (indicated by stars). D, time course of ERK activation after stimulation of quiescent NIH3T3 cells with 10% FBS alone or together with 20 μg/ml cycloheximide (Cx). The upper two panels show Western blot analysis detecting ERK as described above (stars indicate phosphorylated protein forms). The lower two panels show ERK activity assayed by the ability of immunoprecipitated ERK to phosphorylate myelin basic protein.

![Fig. 1. Regulation of MKP-1 mRNA expression in response to specific extracellular stimuli and effect of TPA, anisomycin, and](image-url)
pathway. TPA induced a strong activation of ERK, detected by electrophoretic retardation indicating phosphorylated protein forms (Fig. 1B), with no effect on SAPK activity (Fig. 1C). In contrast, UV-light and anisomycin potently activated p46SAPK and p48SAPK, but induced only a weak and transient stimulation of ERK (Fig. 1A, B and C). These data were confirmed by blotting with anti-phosphotyrosine antibodies for ERK phosphorylation and by measurements of the kinase activity of SAPK toward GST-J un (data not shown).

While TPA activated ERK, it did not induce MKP-1 gene expression. Other groups (15, 16) have reported TPA to be a weak stimulator of MKP-1 mRNA. In contrast, UV-light and anisomycin were potent stimuli of MKP-1 mRNA expression (Fig. 1A). These data suggest that activation of the SAPK pathway by cellular stress induces MKP-1.

In the presence of the protein synthesis inhibitor cycloheximide, serum induced a sustained activation of ERK (Fig. 1D). These data were confirmed using actinomycin D, an inhibitor of transcriptional activity (data not shown). Thus, in accordance with findings by Sun et al. (6), the synthesis of new protein, presumably of a transcriptionally regulated dual specificity protein-tyrosine phosphatase like MKP-1, is required for the inactivation of ERK in NIH3T3 cells.

We used NIH3T3 cells stably transfected with vectors encoding mutants of MEK, the upstream kinase of ERK, in order to investigate the role of the MEK-ERK module in the regulation of MKP-1 (10). As shown previously (10), the constitutively active mutants of MEK exhibit a 55-fold increase in activity compared to wild type, resulting in the morphological transformation of transfected cells (10) and the stimulation of ERK (data not shown). However, cells expressing constitutively active MEK did not show increased gene expression of MKP-1 compared to unstimulated wild-type or neo-transfected cells (Fig. 2A, lanes 10, 1, and 4). In fact, transcription of MKP-1 after stimulation with serum was diminished in wild-type compared to neo-transfected cells and was inhibited in cells expressing constitutively active MEK (Fig. 2A). Moreover, MKP-1 gene expression following stimulation with serum (Fig. 2A) or anisomycin (Fig. 2B) was enhanced in cells expressing catalytically inactive MEK. In accordance with this finding, pre-stimulation of the ERK cascade with TPA potently inhibited the MKP-1 gene expression in response to serum in parental NIH3T3 cells (Fig. 2C). Thus, in these cells in which ERK is selectively regulated by mutants of MEK as well as in the experiments using selective extracellular agonists, MKP-1 gene expression is not induced by activation of ERK. Moreover, from these results, it appears that activation of the MEK-ERK module correlates with an inhibition of MKP-1 gene expression.

Previously, we (3) and others (17, 18, 19) showed that MEK kinase (MEKK) selectively phosphorylates and activates SEK1, which subsequently phosphorylates and activates SAPK. In vivo MEKK is suggested to stimulate SAPK rather than HOG1 kinase (17, 19), a recently identified MAP kinase-like kinase. Stably transfected NIH3T3 cells expressing active MEKK (ΔMEKK) in response to IPTG (3) were used to investigate the effect of the SAPK pathway on MKP-1 expression. As shown previously (3), SAPK activity increased 6- to 8-fold after induction of ΔMEKK for 12 to 23 h in comparison to parental NIH3T3 cells. IPTG did not affect MKP-1 gene expression in parental NIH3T3 cells but induced MKP-1 mRNA in ΔMEKK-inducible cell lines (Fig. 3). These findings, as well as the positive effect of UV-light and anisomycin on MKP-1 gene expression, reinforce the conclusion that activation of the SAPK pathway induces MKP-1.

Previously we have shown that induction of ΔMEKK in NIH3T3 cells inhibits ERK activation in response to TPA (3). In accordance with this finding, we show in Fig. 4A that pre-stimulation with UV-light, a potent stimulus of the SAPK cascade and hence MKP-1 expression (Fig. 1), inhibits the TPA-stimulated ERK activation as detected by the reduced appearance of phosphorylated ERK protein forms and reduced ERK activity. Thus, our data provide in vivo evidence for the regulation of ERK activity by SAPK-induced MKP-1 in response to cellular stress.

Our results demonstrate cross-talk between the Raf-MEK-ERK signaling pathway and the recently identified MEKK-
Induction of MKP-1 by SAPK Cascade

**Fig. 3.** MEKK expression in NIH3T3 cells induces MKP-1. Parental cells and stably transfected cells that express active MEKK in response to IPTG were incubated with 1 mM IPTG for indicated times. MKP-1 mRNA was detected by Northern blot analysis as described above. MKP-1 hybridization signals were quantified by scanning densitometry, normalized to the glyceraldehyde-3-phosphate dehydrogenase signal, and expressed as -fold increases over unstimulated levels.

**Fig. 4.** Cross-talk between the MAP kinase pathways Raf-MEK-ERK and MEKK-SEK-SAPK. A, quiescent parental NIH3T3 cells were prestimulated with UV-light (40 J/m²) for 75 min (+) or untreated (–) prior to stimulation with TPA (100 nM) for the indicated periods of time. The upper panel shows a Western blot analysis detecting p42ERK as described above (stars indicate phosphorylated protein forms), and the lower panel shows ERK activity assayed by the ability of immunoprecipitated ERK to phosphorylate myelin basic protein (MBP). B, we describe the induction of MKP-1, that is capable of inactivating ERK, by the MEKK-SEK-SAPK pathway. Thereby SAPK may control the activity of the functionally antagonistic ERK pathway. Furthermore, we show that activation of the MEK-ERK module correlates with an inhibition of the MKP-1 gene expression (dotted line). SEK-SAPK pathway (2, 3, 17) (Fig. 4B). We show that the expression of MKP-1 is induced by activation of the SAPK pathway but not by stimulation of ERK. These data support the existence of an important mechanism involved in the maintenance of signaling specificity, whereby activation of the MEKK-SEK-SAPK pathway induces a phosphatase that is capable of inactivating the Raf-MEK-ERK pathway. Since activation of ERK is known to mediate cell growth, this cross-talk of MAP kinase cascades could contribute to the growth inhibition observed following increased MEKK activity and SAPK stimulation (3). In addition, our data suggest that activation of ERK may inhibit the induction of MKP-1. This mechanism may contribute to our previously described finding that the expression of constitutively active MEK induces loss of cell growth control in NIH3T3 cells (10). Recently, it was shown that MKP-1 may play a role in the down-regulation of the cellular stress response since transfection of HeLa cells with MKP-1 inhibited the activation of SAPK (20). Therefore, the induction of MKP-1 by SAPK may also be important for the deactivation of SAPK in the way of a classical feedback mechanism. In vitro and in vivo data in rat fibroblasts, however, support a relative selectivity of MKP-1 for ERK over SAPK (7).

Complex factors like serum and growth factors like platelet-derived growth factor or epidermal growth factor induce MKP-1 gene transcription (16). Based on our data, it is likely that growth factors induce MKP-1 expression due to the activation of signaling pathways distinct from the ERK cascade such as the SAPK cascade. Growth factors stimulate ERK (4) and SAPK (21, 22) in a Ras-dependent way (18), whereas SAPK is also inducible in a Ras-independent way by tumor necrosis factor α (18). Therefore, it is likely that the mitogenic and Ras-dependent activation of ERK by growth factors is controlled by a simultaneous stimulation of the SAPK pathway that induces MKP-1 expression (Fig. 4B).

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