MEKK3 Directly Regulates MEK5 Activity as Part of the Big Mitogen-activated Protein Kinase 1 (BMK1) Signaling Pathway*

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Big mitogen-activated protein (MAP) kinase (BMK1), also known as ERK5, is a member of the MAP kinase family whose cellular activity is elevated in response to growth factors, oxidative stress, and hyperosmolar conditions. Previous studies have identified MEK5 as a cellular kinase directly regulating BMK1 activity; however, signaling molecules that directly regulate MEK5 activity have not yet been defined. Through utilization of a yeast two-hybrid screen, we have identified MEKK3 as a molecule that physically interacts with MEK5. This interaction appears to take place in mammalian cells as evidenced by the fact that cellular MEK5 and MEKK3 co-immunoprecipitate. In addition, we show that a dominant active form of MEKK3 stimulates BMK1 activity through MEK5. Moreover, we demonstrate that MEKK3 activity is required for growth factor mediated cellular activation of endogenous BMK1. Taken together, these results identify MEKK3 as a kinase that regulates the activity of MEK5 and BMK1 during growth factor-induced cellular stimulation.

The mitogen-activated protein (MAP) kinase cascades represent a primary mechanism by which cells transduce intracellular signals (1–3). These kinase cascades display a high degree of evolutionary conservation, as evidenced in a variety of eukaryotes ranging from yeast to mammals (2, 4). Three sequentially activated kinases make up the core of the MAP kinase module: a MAP kinase kinase kinase, or MEKK; a MAP kinase; and a MAP kinase (5, 6). In addition to delivering signals from extracellular stimuli to intended effectors, these kinase modules harmonize incoming signals from parallel signaling pathways and provide signal amplification as well as biological specificity. To date, four separate MAP kinases have been identified in mammalian cells and are known as ERK, JNK/SAPK, p38, and BMK1/ERK5 (7–13).

BMK1/ERK5 represents the newest member of the mammalian MAP kinase family and was independently cloned by our laboratory and another group using different experimental approaches (7, 11). We have previously demonstrated that BMK1 is activated by growth factors, oxidative stress, and hyperosmolar conditions (8, 9, 14). Upon activation, BMK1 stimulates the activity of myocyte enhancer factor 2C (MEF2C), a transcription factor that induces the expression of the proto-oncogene c-jun (9). Through the use of a dominant negative form of BMK1, we have demonstrated that BMK1 is required for growth factor-induced cell proliferation and cell cycle progression (8). Using the yeast two-hybrid system, MEK5 was identified by Zhou et al. (11) as the molecule responsible for regulating BMK1 activity. Subsequently, we have determined that MEK5 specifically activates BMK1 but not other mammalian MAP kinases in vivo (9). In addition, we have shown that MEK5 activity is required for the activation of BMK1 induced by extracellular stimuli (8, 9).

The upstream kinase responsible for regulating MEK5 activity within the BMK1 signaling module has not yet been reported. In this regard, studies by English et al. (15) demonstrated that MEKK1, Raf-1, and Mos1 are unable to phosphorylate MEK5 efficiently, indicating that none of these MEKKs are responsible for regulating MEK5 activity. Here, using a yeast two-hybrid screening method, we have identified MEKK3 as a molecule that interacts with MEK5. Through the use of a dominant active form of MEKK3, we show that MEKK3 activates endogenous BMK1 through MEK5. In addition, we also demonstrate that MEKK3 activity is required for EGF-induced cellular activation of BMK1 but not ERK1/2. These results identify MEKK3 as the first kinase activated in the BMK1 signaling module during EGF-induced cellular stimulation.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The full-length cDNA Sma1 to Sali fragment of MEK5(A) was fused in frame with the GAL4 DNA binding domain of pGAL9, resulting in the expression vector pGAL9-MEK5(A). The cDNA library was derived from mouse kidney fibroblasts and kindly provided by Dr. Mary Pauza (The Scripps Research Institute). This library was fused with the activation domain of GAL4 in the plasmid pGAD424 (CLONTECH) and used to search for proteins that interact with MEK5(A). Yeast transformation and two-hybrid screening were performed as described elsewhere (16).

Antibodies—The monoclonal antibody 12CA5, against the hemagglutinin (HA) epitope, was purchased from Babco (Emeryville, CA). The M2 antibody against the FLAG epitope was purchased from Eastman Kodak. Antibodies against MEK5 and MEKK3 were purchased from StressGene (Victoria British Columbia, Canada). Antibodies against BMK1 were made as described (14). Antibodies against ERK and phospho-ERK were purchased from New England Biolabs (Beverly, MA).

DNA Constructs—Enzyme-inactive kinases were obtained by changing the ATP binding sites of the kinases of MEK5 and MEKK3 from Lys-195 to Met-195 and from Lys-393 to Thr-393, respectively. This polymerase chain reaction (PCR)-based mutagenesis (9) resulted in MEK5(M) and MEKK3(W), respectively. The BglII-SalI cDNA fragments of MEK5 and MEKK3(W) and truncated forms of MEK5 were...
inserted into the vector pR5, either alone or fused in frame at the carboxyl terminus with the protein sequence YPYDVPDYAGYPYDVP-DYAGSPYPDVPDYAAPQC that encodes three copies of the HA epitope. Plasmids expressing MEK5, MEK5(D), MEK5(A), BMK1, and BMK1(AEF) have been described previously (9).

**Immunoprecipitation and In Vitro Kinase Assays**— Cells were solubilized in lysis buffer (20 mM HEPES (pH 7.6), 1% Triton X-100, 137 mM NaCl, 0.1 mM Na3VO4, 25 mM β-glycerophosphate, 3 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) for 10 min at 4 °C. Cell lysates were then centrifuged at 15,000 × g for 15 min at 4 °C. HA-tagged protein kinases were immunoprecipitated for 16 h at 4 °C using 12CA5 antibody conjugated to agarose beads. The beads were washed twice with 1 ml of lysis buffer and then washed twice again with 0.5 ml of kinase reaction buffer (20 mM HEPES (pH 7.6), 20 mM MgCl2, 0.1 mM Na3VO4, 25 mM β-glycerophosphate, and 2 mM dithiothreitol). Kinase assays were performed and analyzed as described previously (9).

**RESULTS AND DISCUSSION**

MEKK3 Is the Kinase That Directly Regulates MEK5 Activity— In an effort to identify proteins that interact with MEK5, we utilized a yeast two-hybrid system. To this end, a dominant negative form of MEK5, MEK5(A), was fused in frame to the DNA-binding domain of GAL4 and subsequently used as bait in the yeast two-hybrid system. A mouse embryonic cDNA library was fused with the activating domain of GAL4 and a total of 1.2 × 107 transformants were screened. 57 clones were selected for sequence analysis based on their potential interaction with MEK5 as evidenced by increased β-galactosidase activity. Using the BLAST algorithm and the nucleotide data base at the National Library of Medicine, two of the clones were found to encode partial sequences of MEKK3. These clones, MEKK3213 and MEKK3324, encode amino acids 213–626 and 324–626 of MEKK3, respectively (Fig. 1A). MEK5 is a 626-amino acid kinase that was cloned by PCR using the known sequence for MEKK1 (17) and also by differential display screening (18). As shown in Fig. 1A, both MEKK3213 and MEKK3324 interact with MEK5(A) in the yeast two-hybrid system. The resulting activation of β-galactosidase activity is completely dependent on the presence of sequences from both MEKK3 and MEK5(A) in this GAL4 system and therefore represents a specific interaction between these two proteins.

To verify the results of the two-hybrid screen, it was important to establish whether MEK5(A) and MEKK3 interact in vivo. Therefore, we cloned full-length MEKK3 from cellular mRNA isolated from NIH3T3 cells using reverse transcriptase-PCR. The fidelity of this MEKK3 clone was confirmed by DNA sequencing. The MEKK3 cDNA was subsequently subcloned into a mammalian expression vector to create HA-tagged MEKK3. The resulting vector was cotransfected into 293T cells, with or without an expression vector encoding MEK5(A), followed by the preparation of cell lysates and immunoprecipitation using an anti-HA antibody. MEK5 was detected in the immunoprecipitate only when co-expressed with the HA-tagged MEKK3 in 293T cells (Fig. 1B). These results support the results of the yeast two-hybrid screen and suggest that MEKK3 and MEK5 can physically interact with each other in mammalian cells.

To determine the ability of MEKK3 to phosphorylate MEK5, full-length MEKK3 protein was tested in an in vitro protein kinase assay using recombinant MEK5 as a substrate. Full-length MEKK3 displayed little or no kinase activity for MEK5 (Fig. 2A). In this regard, other studies have demonstrated that the amino-terminal domains of MAP kinase kinase kinases, such as MEKK1 or Raf1, act to self-regulate their kinase activity, and deletion of this amino-terminal regulatory domain has been shown to render these kinases constitutively active (5, 19–22). To determine whether the amino-terminal domain of MEKK3 has a similar role in self-regulation, we constructed MEKK3Δ11 by deleting the first 11 amino acids of this kinase.

This deletion dramatically increased the ability of MEKK3 to phosphorylate MEK5 (Fig. 2A). In addition, we discovered that MEKK3Δ11 autophosphorylates itself. Additional MEKK3 constructs with larger amino-terminal deletions of 213 and 322 amino acids, MEKK3Δ213 and MEKK3Δ322, respectively, also phosphorylated MEK5 but lost their ability to autophosphorylate themselves (Fig. 2A).

Because MEKK3 appears to act as a kinase for MEK5, we wished to determine whether this reaction enables MEK5 to mediate phosphorylation of its downstream substrate BMK1. We have previously established that the mobility of BMK1 in SDS-polyacrylamide gels is significantly retarded upon phosphorylation or activation in comparison with nonphosphorylated or inactive BMK1 (8). Therefore, we co-expressed FLAG-tagged BMK1 along with various forms of MEK5 and/or MEKK3 and examined the mobility of BMK1 in Western blots probed with anti-FLAG antibody (Fig. 2B). As expected, wild type MEK5 alone cannot activate BMK1 in this system. Conversely, a dominant active form of MEK5, MEK5(D), fully
MEKK3 phosphorylates and activates MEK5. A, 293T cells were transiently transfected with expression vectors encoding HA-tagged forms of MEKK3, MEKK3Δ11, MEKK3Δ213, or MEKK3Δ222, as indicated. The upper panel shows the kinase activity of these MEKK3 proteins toward MEK5 as measured in an immune complex protein kinase assay using a kinase-dead form of MEK5, MEK5(M), as a substrate. The lower panel shows the expression of the HA-tagged MEKK3 proteins as detected in the corresponding immunoprecipitates using anti-HA antibody. B, expression vectors encoding FLA7-tagged MEK5 and BMK1 were co-transfected along with control vector pcDNA3 or expression vectors containing HA-MEK5(D), HA-MEK5, MEKK3, or MEKK3Δ11, as indicated in the figure. The top panel shows the mobility of FLAG-tagged BMK1 from the transfected cell lysates detected in a Western blot using anti-FLAG epitope antibody. The middle and bottom panels of the figure show the expression of MEKK3 and HA-tagged MEK5 proteins detected in transfected cell lysates by Western blotting using antibodies against MEKK3 or HA, respectively.

Activated BMK1. Wild type MEKK3 was unable to activate FLA7-tagged BMK1 even when this system was supplemented with wild type MEK5. Conversely, dominant active MEKK3, MEKK3Δ11, fully activated BMK1 but only when co-expressed with wild type MEK5 (Fig. 2B). These results demonstrate that MEK5 is required for the dominant active form of MEKK3 to activate BMK1.

We found that expression of MEKK3Δ11 in 293T cells significantly activated endogenous BMK1 (Fig. 3). To confirm the requirement for MEK5 in MEKK3-mediated activation of BMK1, we cotransfected MEKK3Δ11 with a dominant negative form of MEK5, MEK5(A), in 293T cells. Expression of MEK5(A) blocked the MEKK3Δ11-mediated stimulation of BMK1, confirming that the activation of endogenous BMK1 by MEKK3 is mediated through MEK5. Other studies have shown that activation of MEKK3 leads to phosphorylation of ERK1 and 2 (17, 18), and we observed the same effect using MEKK3Δ11 (Fig. 3). However, although the expression of MEK5(A) blocked the MEKK3Δ11-mediated activation of BMK1, it had no effect on the MEKK3Δ11-mediated activation of ERK (Fig. 3). Thus, MEK5 appears to be a specific signal transducer of MEKK3-induced activation of BMK1.

MEKK3 Mediates EGF-induced BMK1 Activation—We have previously demonstrated that BMK1 is required for EGF-induced cell proliferation (8). In addition, we have shown that MEK5 is a cellular kinase mediating EGF-induced cellular activation of BMK1 (8). As MEKK3 has been identified here as an upstream mediator of the BMK1 kinase cascade, it was important to establish whether this MEKK is involved in EGF-induced activation of the BMK1 signal transduction pathway. As shown in Fig. 4A, MEKK3 is activated in cells after EGF treatment. The activation of MEKK3 was maximal within 5 min after the addition of EGF and remained activated for approximately 30 min (Fig. 4A). To address whether MEKK3 mediates the EGF-induced activation of BMK1, we constructed a kinase-inactive form of MEKK3 by mutating Lys391 to Trp in the ATP binding domain. Expression of MEKK3(W) substantially inhibited EGF-induced stimulation of endogenous BMK1 (Fig. 4B). In addition, the inhibitory effect of MEKK3(W) seemed to be specific for BMK1 activity, as MEKK3(W) expression did not modify the EGF-induced activation of ERKs (Fig. 4B). These results suggest that MEKK3 activity is required for EGF-induced BMK1 activation and that its role in EGF-induced ERK activation is not significant.

Although MEKK3 appears to activate the ERKs, and all of these kinases are activated by EGF, our results indicate that MEKK3 does not mediate EGF-induced activation of the ERKs. In fact, a number of studies have clearly shown that EGF-induced cellular activation of the ERKs is mediated by the
Ras/Raf pathway (23–26). In this regard, we have previously demonstrated that EGF-induced activation of BMK1 occurs independently of the Ras/Raf pathway and is specifically mediated by MEK5 (8). Here, we have identified MEKK3 as a kinase that activates BMK1 through MEK5. Moreover, we have shown that MEKK3 mediates EGF-induced activation of BMK1 but not the ERKs. In addition to demonstrating that EGF-induced activation of BMK1 occurs via MEKK3 and MEK5, our results reinforce the idea that this signal transduction pathway is distinct from that leading to EGF-induced ERK activation. Nevertheless, it remains possible that MEKK3 is involved in ERK activation induced by other agonists or within other cell types. The upstream regulator for MEKK3 has not yet been identified, and although 14-3-3 proteins have been shown to interact with MEKK3, this interaction alone does not appear to affect the activity of MEKK3 (27). Thus, 14-13-3 proteins may act as scaffold proteins to localize MEKK3 and regulate MEKK3 activity through coordination with other signaling molecule(s).

In summary, our findings demonstrate that MEKK3 together with MEK5 and BMK1 constitute a distinct signaling kinase cascade that is activated during growth factor-induced cellular stimulation.

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