MEKK1 Binds Directly to the c-Jun N-terminal Kinases/Stress-activated Protein Kinases*

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Mitogen-activated protein (MAP) kinases mediate responses to a wide array of cellular stimuli. These cascades consist of a MAP kinase or extracellular signal-regulated kinase (ERK), activated by a MAP/ERK kinase (MEK), in turn activated by a MEK kinase (MEKK). MEKK1 has been shown to be a strong activator of the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway. We report here that JNK/SAPK binds directly to the N-terminal, noncatalytic domain of MEKK1 in vitro and in transfected cells. Immobilized MEKK1-derived peptides extract JNK/SAPK selectively from cell lysates. MEKK1 communoprecipitates with multiple JNK/SAPK isoforms in transfected cells. Expression of the N terminus of MEKK1 lacking the kinase domain increases activation of endogenous JNK/SAPK by MEKK1. The data are consistent with a model in which MEKK1-JNK/SAPK binding facilitates the receipt of signals from upstream inputs and localizes JNK/SAPK to intracellular targets of the pathway.

The c-Jun N-terminal-kinase/stress-activated protein kinases (JNK/SAPKs)† were originally discovered as activities enhanced by exposure of cells to cycloheximide (1). Subsequently, they have been shown to be activated in response to numerous environmental stresses, including UV light, osmotic shock, inflammatory cytokines and, to a lesser extent, growth factors (2–6). Some JNK/SAPK isoforms bind tightly to a domain contained within a subset of the Jun family of transcription factors (2–6). Some JNK/SAPK isoforms in transfected cells. MEKK1 coimmunoprecipitates with multiple JNK/SAPK isoforms in transfected cells. Expression of the N terminus of MEKK1 lacking the kinase domain increases activation of endogenous JNK/SAPK by MEKK1. The data are consistent with a model in which MEKK1-JNK/SAPK binding facilitates the receipt of signals from upstream inputs and localizes JNK/SAPK to intracellular targets of the pathway.

Numerous regulators upstream of the MEKs in the JNK/SAPK pathway have been identified although it has been difficult to assess their physiological roles. Protein kinases related to both the enzymes Ste11p and Ste20p of the pheromone response pathway of budding yeast (12) activate JNK/SAPK in transfected cells or Xenopus oocyte extracts. These include MEKK1–4, germinal center kinase, kinases responsive to transforming growth factor β, tumor necrosis factor-α, the p21-activated kinases (PAKs), mixed lineage kinases, and the Nek-interacting kinase (NIK) (13–24). Some of these kinases phosphorylate MEKs, and the others are presumed to lie further upstream. Most of these upstream regulators of the JNK/SAPK pathway appear to be constitutively active. MEKK1 was the first of these to be identified (13, 15). In vitro, it phosphorylates several MEK family members that lead to activation of not only JNK/SAPKs but also ERKs and p38 in reconstituted systems (25); however in most transfected cells, MEKK1 stimulates cotransfected JNK/SAPK preferentially (15, 16, 26). The molecular mechanisms underlying the restricted specificity of MEKK1 in vivo are unexplored.

By cDNA cloning, we determined that MEKK1 contains a long, noncatalytic N terminus of nearly 1200 residues with several potential protein-association domains (15). This N-terminal region binds to putative upstream kinases, such as NIK (20). In this report, we assessed the ability of this portion of MEKK1 to bind to a downstream MAP kinase, JNK/SAPK.

**EXPERIMENTAL PROCEDURES**

Plasmids—pGEX-KX/MEKK1 30–220 was constructed by ligation of a 575-base pair SacI fragment of MEKK1 encoding residues from 30 to 220 to SacI-digested pGEX-KX, which was modified from pGEX-KG (Invitrogen). pGEX-KG/MEKK-C was constructed by ligation of a Ncol-Xhol fragment of rMEKK1 that covers the entire catalytic domain to pGEX-KG digested with Ncol and Xhol. pC PIPEHA/MEKK-N was constructed by deleting a 1.4-kilobase EcoRV-BamHI fragment encoding the kinase domain from pCEP4HA/rMEKK1. pCEP4HA/p38 and pCMV5/MEKK1 were constructed as described (15). pCMV5HA/ rMEKK-C was provided by Jeff Frost University of Texas Southwestern Medical Center.

Cell Culture and Preparation of Cell Lysates—293 cells (human embryonic kidney cells) and Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. 293 cells were transfected and lysed in 20 mM Tris-Cl, pH 7.6, 1 mM EGTA, 60 mM β-glycerophosphate, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin and aprotinin if not specifically indicated. Confluent MDCK cells were starved in serum-free medium for 20–24 h and exposed to ultraviolet (UV)-C for 2 min at 1 J/m2. Cells were incubated at 37 °C in serum-free medium for 1 h and then lysed in 20 mM Tris-Cl, pH 7.6, 1 mM EGTA, 60 mM β-glycerophosphate, 10 mM MgCl2, 1% Triton X-100, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin and aprotinin. Cellular debris was removed by centrifugation in a microfuge at 4 °C. The supernatants were used for immune complex kinase assays or Western blots.

Immunoprecipitation and Kinase Assays—Cell lysates were incubated with appropriate antibodies and protein A-Sepharose at 4 °C overnight with constant rotation. The beads were washed twice with...
0.25 M Tris-Cl, pH 7.6, 0.5 M NaCl, and once with 0.25 M Tris-Cl, pH 7.6, 0.1 M NaCl. To assay the activities associated with MEKK1 peptides, cell lysates were incubated with peptide-coupled Sepharose. MEKK1 peptides (P1, EWLERRNRRGPVVVKPIPIK; P3, TPPRRAPSPDPSPYSPEET; P4, LSPGLRDVAVRCLELQPQDR; and P5, RADWRRQQLRKVRSVELD) were coupled to CNBr-activated Sepharose (Sigma) as described (15). Sepharose subjected to the same coupling procedure without peptide was used as a control. 300 mg of lysate protein was incubated with Sepharose beads at 4 °C overnight with constant rotation. The beads were washed twice with 0.25 M Tris-Cl, pH 7.6, 0.5 M NaCl, and once with 0.25 M Tris-Cl, pH 7.6, 0.1 M NaCl. c-Jun kinase activity associated with beads was detected by a standard kinase assay. In the inhibition experiments, 200 μg of lysate protein from UV-stimulated MDCK cells was incubated with soluble peptides at concentrations of 0, 0.63, 1.25, 2.5, or 5 mg/ml (0–2.3 mM) at 4 °C for 5 h. Then, P1-Sepharose was incubated with the lysates at 4 °C overnight. The beads were washed four times with cold lysis buffer for 20–30 min at 4 °C with constant rotation. In Vitro Binding Assay—10 μg of GST, GST-rMEKK1 30–220, or GST-rMEKK-C (1175–1493) was immobilized on GSH-agarose in the presence of 10 mg/ml bovine serum albumin, then incubated with Hisc-SAPKα that recognize both 46- and 54-kDa JNK/SAPK isoforms. C, endogenous c-Jun kinase activity in lysates from UV-stimulated MDCK cells selectively associates with MEKK-P5 or P1 peptide-coupled Sepharose. Relative c-Jun kinase activity was determined by comparison to JNK/SAPK activity immunoprecipitated by O976. D, soluble P1, not P5, peptide inhibits the association of c-Jun kinase activity with P1-Sepharose. c-Jun kinase activity associated with P1-Sepharose in the absence or presence of different MEKK1 peptides at the indicated concentrations was detected by phosphorylation of GST-c-Jun (1–122) as described (15). Relative c-Jun kinase activity was determined by comparison to the activity associated with P1-Sepharose in the presence of soluble peptides at 0.63 mg/ml.
JNK/SAPK polyclonal antiserum (Fig. 1B). Activity associated with the P5-beads following incubation with lysates of UV-exposed cells was nearly 60-fold greater than activity on P5-beads incubated with lysates of untreated cells, consistent with the extent of activation of JNK/SAPK by exposure to UV light. A second peptide P1 also bound c-Jun kinase activity in lysates (Fig. 1C) as effectively as P5, but other MEKK1 or irrelevant control peptides or beads coupled without peptide did not (Fig. 1, B and C). c-Jun kinase activity bound to P1- or P5-Sepharose was not removed with 0.5 M NaCl but could be competed with unbound peptide; soluble P1 reduced c-Jun kinase activity associated with P1-beads in a concentration dependent manner, but P3 peptide was a poor competitor of this binding (Fig. 1D).

Endogenous JNK/SAPK Isoforms Bind to MEKK1 Peptides—The properties of the c-Jun kinase activity suggested that it was derived from JNK/SAPK. To determine if JNK/SAPK accounted for the activity bound to the beads, proteins associated with MEKK1 peptide-coupled beads were immuno-blotted with an anti-JNK/SAPK antiserum (Fig. 2). Both 54 and 46 kDa isoforms of JNK/SAPK were detected on P5-beads incubated with stimulated or control lysates. When the antiserum was incubated with recombinant SAPK protein, the immunoreactive bands (p54 and p46) disappeared, indicating that they represent JNK/SAPK isoforms (Fig. 2A).

JNK/SAPKs also bound to P1-beads but not P3-beads or beads without peptide, in agreement with the activity assays (Fig. 2B). Little or no immunoreactivity was detected on the beads with antibodies to ERKs or p38, under conditions where signals from all three types of kinases in cell lysates were nearly equivalent (data not shown). In addition, no detectable MEK or MEKK activities were detected on P1- or P5-coupled Sepharose following incubation with lysates (not shown).

Consistent with the activity assay (Fig. 1D), the JNK/SAPK bands associated with P1-beads were lost upon incubation with soluble P1 as the competitor but were retained during incubation with P3 as the competitor (Fig. 2C). The association of JNK/SAPK with MEKK peptides is likely direct since recombinant SAPK-a1 binds to P1- and P5-Sepharose (not shown). Therefore, the association of c-Jun phosphorylating activity with peptide-coupled beads parallels JNK/SAPK binding. This supports the conclusion that JNK/SAPK accounts for the UV-stimulated, c-Jun kinase activity associated with P1- and P5-coupled beads.

JNK/SAPK Binds and Phosphorylates a MEKK1 Fragment In Vitro—In vitro recombinant SAPK-a1 bound to a fragment of MEKK1 that includes the P1 and P5 peptides (residues 30–220; Fig. 3A) but not to a fragment (residues 1175–1493) containing only the protein kinase domain. In examining the interaction of JNK/SAPK with MEKK1 fragments, we found that the JNK/SAPK-binding fragment, residues 30–220, was highly phosphorylated by activated SAPK-a1 in vitro (Fig. 3B), although peptides P1 and P5 themselves did not contain any candidate JNK/SAPK phosphorylation sites. The phosphorylation of the JNK/SAPK binding fragment of MEKK1 by JNK/SAPK further suggests that the two proteins interact in cells. Phosphorylation of MEKK1 by JNK/SAPK may have a regulatory impact if, for example, phosphorylation alters the affinity of the JNK/SAPK-MEKK1 association. However, under the conditions of our assays, we detected no difference in their association as a function of JNK/SAPK activation state.

JNK/SAPK Coimmunoprecipitates with MEKK1 in 293 Cells—The capacity of MEKK1 and JNK/SAPK to coimmunoprecipitate was evaluated. MEKK1 was expressed alone or with HA-JNK1 (Fig. 4A). As a control, HA-p38 was coexpressed with MEKK1. MAP kinases were immunoprecipitated with the anti-HA antibody. MEKK1 was detected in anti-HA immunoprecipitates, if HA-JNK1 was expressed, but not in the absence of HA-JNK1. Negligible coimmunoprecipitation of MEKK1 was detected in anti-HA immunoprecipitates if HA-p38 was coexpressed with it (Fig. 4A). We further investigated whether the N-terminal region of MEKK1 coprecipitates with JNK/SAPK.
The N terminus of MEKK1 (HA-MEKK-N), residues 1–1197, was expressed separately or with GST-SAPK\(\beta\) in 293 cells (Fig. 4B). Transfected cells were lysed, and either MEKK-N was immunoprecipitated with an anti-HA monoclonal antibody or GST-SAPK\(\beta\) was precipitated with glutathione beads. Coprecipitated proteins were analyzed by probing with anti-MEKK1 or anti-JNK/SAPK antibodies. MEKK-N was bound to glutathione beads when coexpressed with GST-SAPK\(\beta\) but not in the absence of GST-SAPK\(\beta\) (Fig. 4B, lower panel). GST-SAPK\(\beta\) was present in the anti-HA immunoprecipitates only if HA-MEKK-N (Fig. 4B, upper panel) was expressed. Thus, multiple isoforms of JNK/SAPK, but not p38, are associated with MEKK1 in transfected 293 cells. A catalytic domain fragment of MEKK1 neither coimmunoprecipitates with JNK/SAPK from transfected cells (not shown) nor binds to JNK/SAPKs in vitro (Fig. 3A), indicating that it does not contain a JNK/SAPK binding domain.

The N Terminus of MEKK1 Activates JNK/SAPK in 293 Cells—Since JNK/SAPK binds to the N terminus of MEKK1, we compared the capacities of transfected MEKK1 and its catalytic domain, MEKK-C, to activate endogenous JNK/SAPKs (Fig. 5A). Measurable activation of JNK/SAPK was detected in immune complex assays upon transfection of 50 ng of DNA of either MEKK1 or MEKK-C; however, equivalent or greater increases were induced by MEKK-C at each DNA amount (Fig. 5A). Western blotting the lysates with anti-active JNK/SAPK confirmed these findings (Fig. 5B). MEKK1 and MEKK-C also activate coexpressed JNK1 equally well (data not shown).

To explore the possible function of the observed association, MEKK-N was expressed in 293 cells to determine its effects on the activity of endogenous JNK/SAPK. Interestingly, MEKK-N alone caused a dose-dependent increase in JNK/SAPK activity present in anti-JNK/SAPK immune complexes (Fig. 5C). Similar results were obtained when MEKK-N was cotransfected with HA-JNK1 (not shown). In addition, cotransfection of MEKK-N with small amounts of full-length MEKK1 potentiated JNK/SAPK activation. Activity of endogenous JNK/SAPK (Fig. 5C) or cotransfected HA-JNK1 (not shown) was greater than that caused by the sum of either MEKK-N or full-length MEKK1 alone. These findings suggest a role for the N terminus of MEKK1 in regulating the JNK/SAPK pathway although it is not required for JNK/SAPK activation when MEKK1 is overexpressed.

DISCUSSION

We have demonstrated that the N-terminal domain of MEKK1 binds directly to its downstream MAP kinase, JNK/SAPK. MEKK1-derived peptides extract JNK/SAPK activity from cell lysates as effectively as anti-JNK/SAPK antibodies. JNK/SAPK binds to an N-terminal fragment (residues 30–220) of MEKK1 in vitro. Transfected MEKK1 coimmunoprecipitates with multiple JNK/SAPK isoforms in cells. In comparison, p38 MAP kinase interacts poorly with MEKK1, indicating the specificity of MEKK1 association with JNK/SAPKs.

Complex formation exists widely in signaling processes and has been found to be an important mechanism to facilitate signal transduction and maintain signaling specificity (27–29). In the pheromone mating factor pathway of budding yeast,
three kinases in the MAP kinase module, Ste11, Ste7, and Fus3/Kas1, form a complex through binding to a scaffolding protein, Ste5p. This is essential to the function of the pathway. Our data indicate the existence of a complex between MEKK1 and a MAP kinase in mammalian cascades, which is likely to be important for the function of the cascade. Since the C-terminal half of MEKK1 also binds to MEK4/SEK1, it will be interesting to investigate whether the three kinases in the MAP kinase module form a complex simultaneously.

In transfected cells, the association of MEKK1 and JNK/SAPK is not required for JNK/SAPK activation since the kinase domain alone, which does not bind to JNK/SAPK, activates JNK/SAPK as well as does full-length MEKK1. However, under limiting conditions within the cell, the binding of endogenous JNK/SAPK to endogenous MEKK1 may be essential for activation of the pathway by MEKK1. Interestingly, we found that the N terminus of MEKK1 causes a small but significant activation of endogenous JNK/SAPK. Because the transfection efficiency is approximately 20%, the fold activation of JNK/SAPK by MEKK-N is underestimated. The mechanism by which the N terminus of MEKK1 increases JNK/SAPK activity is not clear at present. One possibility is that MEKK-N binds and colocalizes a portion of endogenous JNK/SAPK with upstream regulators which activate them. A previous study has shown that MEKK1 binds a putative upstream kinase, NIK, at its N terminus (20). We also find that the N terminus of MEKK1 binds to α-actinin, an actin-binding protein. Apparently, the long, non-catalytic region at its N terminus provides binding sites for several proteins with different functions. Perhaps the function of the association of MEKK1 with multiple kinases in the cascade is to hold different components together to facilitate signal transduction and to localize a portion of these kinases to a particular site in cells. For example, the binding of the total JNK/SAPK to MEKK1 may direct a small fraction of JNK/SAPK to the cytoskeleton, among the presumed intracellular targets of the JNK/SAPK pathway. Interaction of MEK4/SEK1 with another actin-binding protein ABP280 has been reported to be important for activation of the JNK/SAPK pathway by tumor necrosis factor-α (30). Apparently, the N terminus of MEKK1 is involved in many binding events with both upstream and downstream kinases as well as proteins outside the cascade. Elucidation of the function and physiological significance of these binding events will be extremely important to understand the function and regulation of MEKK1.

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