MEKK1 Binds Raf-1 and the ERK2 Cascade Components*

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Mitogen-activated protein (MAP) kinase cascades are involved in transmitting signals that are generated at the cell surface into the cytosol and nucleus and consist of three sequentially acting enzymes: a MAP kinase, an upstream MAP/extracellular signal-regulated protein kinase (ERK) kinase (MEK), and a MEK kinase (MEKK). Protein-protein interactions within these cascades provide a mechanism to control the localization and function of the proteins. MEKK1 is implicated in activation of the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and ERK1/2 MAP kinase pathways. We showed previously that MEKK1 binds directly to JNK/SAPK. In this study we demonstrate that endogenous MEKK1 binds to endogenous ERK2, MEK1, and another MEKK level kinase, Raf-1, suggesting that it can assemble all three proteins of the ERK2 MAP kinase module.

Mitogen-activated protein (MAP)1 kinases mediate responses to a wide array of cellular stimuli (1, 2). MAP kinases lie in a three-kinase module consisting of a MAP kinase or extracellular signal-regulated kinase (ERK) activated by a MAP/ERK kinase (MEK) activated by a MEK kinase (MEKK). These modules are controlled by upstream protein kinases, small and heterotrimeric G proteins, and other regulatory mechanisms. Nearly 20 mammalian MAP kinases have been identified that compose at least six modules. Among these the stress response pathways mediated by the MAP kinases c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) and p38 MAP kinases, and the ERK1/2 pathway often coupled to proliferation and differentiation of cells have been most thoroughly studied.

The specificities of protein kinases in vitro are often broader than their functions in cells. For example, phosphoinositide-dependent protein kinase 1 will phosphorylate several protein kinases including cAMP-dependent protein kinase (PKA) on their activation loops to stabilize or activate them; however, PKA is still phosphorylated on this site in phosphoinositide-de-
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FIG. 1. Transfected HA-MEKK1 communoprecipitates with transfected Raf-1 in 293 cells. pCEP4-HA-MEKK1 was cotransfected into 293 cells with either Raf-1, GST, Myc-PAK1, or Myc-TAO2. Raf-1, GST, PAK1, and TAO2 were immunoprecipitated (IP) with antibodies against Raf-1, GST, or Myc, respectively. The immunoprecipitates were then immunoblotted (IB) for the presence of HA-MEKK1 using anti-HA antibodies (top panel). Anti-HA blots of the cell lysates are shown in the middle panel. Anti-Raf-1, GST, and Myc blots of the immunoprecipitates are shown in the bottom panel. One of seven experiments is shown.

FIG. 2. MEKK1 associates with Raf-1 in an activation-state independent manner. pCMV5-Myc-MEKK1 or MEKK1 D1369A were cotransfected into 293 cells with either Raf-1, Raf-1 S259D, or Raf-1 BXB. Overexpressed Raf-1 proteins were immunoprecipitated (IP), and the immunoprecipitates were immunoblotted (IB) for Myc-MEKK1 (top panel). Blots of immunoprecipitates and lysates show the expression levels of transfected proteins are shown in the two lower panels. One of three experiments is shown.

Experimental Procedures

Plasmids—MEKK1 fragments 30–220 and 221–559 were prepared from bacteria as described (28). MEKK1 constructs were generated using polymerase chain reaction with cDNA encoding the full-length wild type protein in pCMV5-Myc. A point mutation in MEKK1, D1369A, renders the kinase inactive by disrupting the conserved aspartic acid residue responsible for binding Mg\(^{2+}\) and was generated as described (32). Mutation of serine 259 to aspartate in Raf-1 is believed to result in an active kinase by eliminating an inhibitory site for 14-3-3 binding, and Raf-1 BXB lacks a portion of its regulatory N terminus and is described (44). All other Raf-1 constructs were generated using polymerase chain reaction with pCMV5-Raf-1 as template. To express Raf-1 in bacteria, its cDNA was inserted at the EcoRI-XhoI sites into pcAL-n (Stratagene), which will incorporate a calmodulin-binding protein fragment at the N terminus of the expressed protein. cDNAs encoding MEK1 and ERK2 were cloned into pCEP4/Ha as described (45).

Cell Culture and Preparation of Cell Lysates—293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Transfected cells were harvested after 48 h and lysed in 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 80 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 7 μg/ml aprotinin. The cells had been starved for 24 h in serum-free medium prior to harvest. Jurkat T cells were grown in RPMI (HyClone) containing 10% fetal bovine serum, harvested at a density of 10^6 cells/ml, and lysed in hypotonic buffer containing 20 mM HEPES, pH 7.6, 10 mM NaCl, 1.5 mM MgCl\(_2\), 1 mM EDTA, 1 mM EGTA, 80 mM β-glycerophosphate, and 1 mM sodium orthovanadate. Nuclear fractions were sedimented at 200 × g. The supernatants were adjusted to a final salt concentration of 1 × NaCl and homogenized using a Dounce homogenizer. After 30 min on ice, the supernatants used for immunoprecipitation were collected by ultracentrifugation for 30 min at 100,000 × g.

Immunoprecipitation—293 cell lysates (0.5 ml at 3–4 mg/ml) were incubated with appropriate antibodies and 30 μl of protein A-Sepharose at 4 °C for 2 h with constant rotation. The beads were washed three times with 1 ml of 293 lysis buffer for a total of 3 h. Immunoprecipitates from Jurkat cells were washed for 2 h in serum-free medium prior to harvest. Jurkat T cells were grown in RPMI (HyClone) containing 10% fetal bovine serum, harvested at a density of 10^6 cells/ml, and lysed in hypotonic buffer containing 20 mM HEPES, pH 7.6, 10 mM NaCl, 1.5 mM MgCl\(_2\), 1 mM EDTA, 1 mM EGTA, 80 mM β-glycerophosphate, and 0.5% Triton X-100. Immunoprecipitates were then blotted for the indicated proteins. Antibodies recognizing the indicated molecules were as follows: MEKK1, C22 (Santa Cruz); Raf-1, SC-133 (Santa Cruz); MEK1, A2227 (45); ERK2, A249/p42 (46); ERK2, pTepY (NEB); p38, Sc-535 (Santa Cruz); ERK3, A654 (47); HA, 12 CA-5 (Babco); and Myc (Cell Culture Center).

Protein Purification—MEKK1 fragments 30–220 and 221–559 were purified as glutathione S-transferase (GST) fusion proteins. Bacteria were induced using 100 μM isopropyl-1-thio-β-d-galactopyranoside for 8 h at 30 °C. Cells were lysed using chicken lysozyme, and DNase was added to degrade DNA. The recombinant baculovirus expressing Raf-1
containing a FLAG epitope was kindly provided by D. Morrison (NIH, Frederick). Purification of Raf-1 from Sf9 cells was performed as described (48). Raf-1 was purified from *Escherichia coli* strain BL21DE3pLys. The cells were induced for 4 h at room temperature, harvested, and lysed as above in five volumes of 50 mM Tris-Cl, pH 8, 0.15 M NaCl, 1 mM magnesium acetate, 10 mM β-mercaptoethanol, 0.5 mM imidazole, 2 mM CaCl₂, 30% glycerol, and 0.1% Triton X-100. The lysate was clarified by sedimentation, and the supernatant was applied to calmodulin affinity resin. Raf-1 protein was eluted with 50 mM Tris-Cl, pH 8, 0.15 M NaCl, 10 mM β-mercaptoethanol, 30% glycerol, 0.1% Triton X-100, and 2 mM EGTA.

**In Vitro Binding Assay**—MEKK1 fragments (0.39 mg) were immobilized on 30 μl of glutathione-agarose in the presence of 10 mg/ml bovine serum albumin and then incubated with 0.34 mg FLAG-Raf-1 or CAL-Raf-1 in 0.2 ml of lysis buffer with 1% Triton X-100 for 2 h at 4°C. Beads were washed three times with 1 ml of 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, and 0.5% deoxycholate for a total of 6 h.

**Kinase Assays**—Immunoprecipitates were washed as described above, resuspended in water, and incubated with 7 μg of purified MEK4 K131M along with 5 μCi of [γ-32P]ATP in kinase buffer (20 mM HEPES, pH 7.8, 10 mM ATP, 10 mM MgCl₂, and 10 mM β-glycerophosphate) for 30 min at 30 °C. Incorporation of radioactive phosphate was determined by autoradiography and liquid scintillation counting of excised bands.

**RESULTS**

We and others previously showed that MEKK1 acts as a scaffold for the JNK/SAPK pathway by binding directly to JNK/SAPK isoforms (28, 29). Ste5p binds the kinases of the yeast MAP kinase module in the pheromone response pathway (10–12). Ste5p also binds to subunits of the heterotrimeric G protein that activates this pathway (49–52). Because the subunits also bind Ste20p, the kinase upstream of the MAP kinase module, they link Ste20p to the scaffolded module (53). Ste20p then phosphorylates the MEKK Ste11p, thus leading to activation of the kinase cascade (54). To test the possibility that MEKK1 may be regulated in a comparable manner, we examined its ability to bind the p21-activated protein kinase, PAK1, another relative of Ste20p (27), and Raf-1 the MEKK in the ERK1/2 pathway. As shown in Fig. 1, neither PAK1 nor TAO2 coimmunoprecipitated with cotransfected MEKK1. However, to our surprise, Raf-1 was readily detected as a MEKK1-associated protein. We estimate that 50% of the overexpressed MEKK1 was bound to Raf-1. The observation that PAK1, TAO2, and the GST control failed to coimmunoprecipitate MEKK1 supports the idea that Raf-1 binding is not due to a nonspecific association.

We next ascertained whether the interaction of MEKK1 with Raf-1 is regulated in a manner dependent upon the activation state of either enzyme. Wild type MEKK1 that is transfected into cells is active even in the absence of a stimulus (32); thus we tested the effect of MEKK1 activity by comparing the amount of Raf-1 coprecipitating with wild type and kinase-dead MEKK1 (D1369A). Fig. 2 shows that Raf-1 coimmunoprecipitates equally with both wild type and kinase-dead MEKK1. In contrast to MEKK1, wild type Raf-1 has low activity when it is expressed in serum-starved cells; thus, to test the effect of stimulated with colchicine (10 μM, 1 h), nocodazole (5 μM, 1 h), sorbitol (0.5 M, 30 min), or left unstimulated. Cells were collected and lysed in hypotonic buffer. Endogenous MEKK1 was immunoprecipitated (IP) and subjected to kinase assay using catalytically inactive MEK4 produced in bacteria as substrate. MEKK1 immunoprecipitates were blotted for Raf-1. MEKK1 immunoblots (IB) show the levels of endogenous protein in the immunoprecipitates. One of five experiments is shown. B, same experiment as shown in A using 293 cells. One of ten experiments is shown.
Raf catalytic activity on the Raf-MEKK1 association, we compared the amount of MEKK1 coprecipitating with the activated Raf-1 mutants, Raf-1 BXB and Raf-1 S259D, and the inactive, wild type protein. All three forms of Raf-1 were present in MEKK1 immunoprecipitates (Fig. 2), indicating that MEKK1 interacts with Raf-1 in a manner independent of the activation state of Raf-1. These findings suggest that Raf and MEKK1 may be associated in cells whether the enzymes are activated or inactive. The data also support the notion that at least a portion of Raf-1 in a cell is constitutively associated with MEKK1. In other experiments, we estimated that the abundance of MEKK1 is close to or less than that of Raf-1.2

To support the conclusion that the MEKK1-Raf-1 interaction has relevance in cells, we probed the potential association of the endogenous proteins. Raf-1 was found in immunoprecipitates of MEKK1 native to Jurkat or 293 cells (Fig. 3). Control antibodies neither immunoprecipitated MEKK1 nor coimmunoprecipitated Raf-1, indicating that the interaction is not the result of protein aggregation or nonspecific trapping of Raf-1 in the precipitates (Fig. 3). MEKK1 immunoprecipitates were washed more stringently with high salt and with detergent to assess qualitatively the strength of the interaction. Neither 1 M NaCl nor 0.1% SDS were sufficient to disrupt the interaction (not shown). These data demonstrate that endogenous MEKK1 and Raf-1 indeed interact strongly, confirming findings with transfected proteins.

We also examined the possible regulation of the MEKK1-Raf-1 interaction by modulating the activation states of the endogenous enzymes. Activities of the kinases were measured in immune complexes using kinase-inactive MEK4 (K131M) and MEK1 (K97M) as substrates for MEKK1 and Raf-1, respectively. Endogenous MEKK1 is inactive in unstimulated Jurkat T cells and is activated by nocodazole, colchicine, and sorbitol (5) (Fig. 3A). Raf-1 is present in MEKK1 immunoprecipitates regardless of the activation state of MEKK1. In numerous experiments there was little or no change in the amount of Raf-1 in the MEKK1 precipitates, although a small decrease was occasionally observed with sorbitol. To test the effect of Raf-1 activity on its association with MEKK1, we used serum-starved 293 cells, because Jurkat T cells did not withstand serum starvation in our hands. Raf-1 is active in cycling Jurkat T cells and in unstarved 293 cells; its activity is reduced by serum deprivation (data not shown). A population of endogenous Raf-1 interacts with endogenous MEKK1 in serum-starved or stimulated 293 cells (Fig. 3B), demonstrating that their association is independent of the activation state of either enzyme.

We next mapped the interacting domains by cotransflecting plasmids encoding each kinase with fragments of the other. The binding site on MEKK1 was first narrowed to a fragment including residues 221–559; the N-terminal region (20–330) and more C-terminal fragments do not interact (Fig. 4A). A fragment containing residues 221–370 still binds to Raf-1, but residues 371–559 do not bind, indicating that residues 221–370 comprise the Raf-1 binding domain. This portion of the regulatory N terminus of MEKK1 includes a proline-rich region, but

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the requirement of individual residues for Raf-1 interaction was not explored. Similar experiments with the N- and C-terminal domains of Raf-1 indicate that the C-terminal half, which contains the catalytic domain of Raf-1, residues 303–648, is sufficient to bind to MEKK1 (Fig. 4). Both Raf-1 and MEKK1 bind a variety of proteins within cells. Thus, it is possible that the interaction between these kinases is mediated by one or more accessory proteins. To test this possibility, GST-MEKK1 fragments and Raf-1 linked to a fragment of a calmodulin-binding protein were expressed and purified from bacteria as fusion proteins. The catalytic domain of Raf-1 was previously expressed as a soluble protein in bacteria using this fusion system (58). In accord with the data from transfected 293 cells, MEKK1 fragment 221–559 bound specifically to purified Raf-1 (Fig. 5). This result demonstrates that MEKK1 binds Raf-1 directly, because no other eukaryotic proteins were present in these preparations. Direct binding was also found using full-length Raf-1 expressed in Sf9 cells (not shown).

Having established that Raf-1 and MEKK1 are associated to a significant extent under all conditions examined, we wished to gain additional evidence supporting the significance of their association. Because MEKK1 can activate the ERK1/2 pathway, we tested the possibility that MEKK1 also binds to MEK1 and ERK2. 293 cells were transfected with HA-tagged MEK1 alone or together with plasmids encoding Myc-tagged fragments or full-length MEKK1. The MEKK1 proteins were immunoprecipitated using the anti-Myc antibody, and precipitates were probed for MEK1. MEK1 does indeed associate with MEKK1, both full-length and an N-terminal fragment, residues 1–482 (not shown). For comparison we examined the ability of MEKK1 to interact with MEK7 of the JNK/SAPK pathway. MEK7 binds to a fragment containing residues 30–400 (not shown).

We showed previously that JNK associates directly with MEKK1 but p38 does not (28). Purified MEKK1 N-terminal fragments and ERK2 both expressed in bacteria bind in vitro (data not shown). To test the ability of MEKK1 to bind to ERK2 in intact cells, 293 cells were transfected with full-length MEKK1, the kinase domain, or several N-terminal fragments either alone or together with HA-tagged ERK2. ERK2 associates with residues 370–559 (Fig. 6), near but distinct from the Raf-1 binding site and not overlapping with the JNK/SAPK binding site located in a D domain-like region within residues 30–220 (28). These findings indicate that MEKK1 can bind components of two MAP kinase modules with selectivity.

To assess the presence of complexes of MEKK1 with the ERK2 MAP kinase module in cells, we first ascertained whether endogenous ERK2 and endogenous MEK1 were associated with overexpressed MEKK1. 293 cells were transfected with MEKK1 or WNK1, another Ste20p-related protein kinase (59), as a control for binding specificity. Endogenous MEK1 and ERK2 were immunoprecipitated individually, and the immunoprecipitates were immunoblotted for transfected MEKK1 and WNK1; Fig. 7A shows the presence of MEKK1 but not WNK1 in MEK1 immunoprecipitates. Fig. 7B shows that endogenous ERK2 immunoprecipitates also contain transfected MEKK1. On the other hand, MEKK1 failed to coimmunoprecipitate with the related MAP kinase ERK3 (Fig. 7B), further supporting the specificity of the association of MEKK1 with ERK2.

To confirm these interactions, endogenous MEKK1 was im-
munoprecipitated from Jurkat cells, and the precipitates were blotted for the presence of ERK2, MEK1, and p38. As shown in Fig. 8A, endogenous ERK2 and MEK1 but not p38 are present in MEKK1 immunoprecipitates. Unlike the Raf-1-MEKK1 interaction, association of ERK2 and MEK1 with MEKK1 is more susceptible to vigorous washing conditions. Immunoprecipitates shown in Fig. 8A were washed using 0.3 M NaCl and 0.1% Triton X-100. A greater concentration of salt or detergent disrupts the interactions.

Finally, we tested the ability of MEKK1 to scaffold an activated ERK2 kinase module. Detection of the active signaling complex would be consistent with the notion that MEKK1 is functioning as a scaffold to maintain signaling specificity. However, we expected the association between MEKK1 and the active kinases to be difficult to detect because they may also dissociate from the scaffold upon activation and move to other cellular compartments. Active MAP kinases, for example, may translocate to the nucleus (1, 2). For this reason, we decided to probe immunoprecipitates of endogenous MEKK1 from cells stimulated with nocodazole, taxol, or sorbitol. All three will activate MEKK1 and sorbitol activates ERK2 (5, 60). To detect ERK2 activity in MEKK1 immunoprecipitates, the precipitated proteins were immunoblotted with antibodies that recognize...
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the active, phosphorylated form of ERK2 (anti-pTyrp) (Fig. 8B). Immunoreactive, active ERK2 was present in the MEKK1 immunoprecipitates from sorbitol-stimulated cells. Longer exposures also revealed some active ERK2 in precipitates from cells treated with nocodazole. MEK1 activity in the MEKK1 immunoprecipitates was assayed using kinase-defective ERK2 (K52R) as substrate. As with ERK2, MEK1 activity was readily detected in the MEKK1 precipitates from sorbitol-stimulated cells (Fig. 8B). If the immunoprecipitates were washed with buffer containing higher salt, the coimmunoprecipitating activities were lost. This suggests that the interaction between MEKK1 and active ERK2 and MEK1 is weaker than the association between MEKK1 and inactive ERK2 and MEK1. Several independent experiments have demonstrated that active endogenous Raf-1 also associates with MEKK1 (data not shown).

DISCUSSION

We show here that MEKK1 scaffolds the ERK2 cascade by binding to each of the three protein kinases of the module, Raf-1, MEK1, and ERK2. The association of these proteins can be readily demonstrated by coimmunoprecipitation of the four endogenous protein kinases. The association of MEKK1 with ERK2 is direct because it can be detected in vitro using proteins expressed in bacteria. The interaction with Raf-1 is direct because bacterially purified Raf-1 binds tightly to MEKK1 fragment 221–559 also purified from bacteria. The positions of the binding sites on MEKK1 suggest that the three kinases may be closely packed on the MEKK1 surface. Because MEK1 binds to both Raf-1 and ERK2 (20, 61), the proximity of their binding sites on MEKK1 might allow these kinases to interact with each other, thereby stabilizing the heteromeric MEKK1 scaffolded complex. MEKK1 like Raf-1 is less abundant than MEK1 or ERK2 (62), indicating that only a small fraction of the downstream kinases are associated with MEKK1 at any given time.

MEKK1 also binds kinases of the JNK/SAPK module (28, 29). The two MEKs that activate JNK/SAPK both bind MEKK1, although apparently on different sites. MEK7 binds MEKK1 outside its kinase domain, whereas Xia et al. (29) reported binding of MEK4 to its kinase domain. MEK4 and MEK7 have at least some distinct functions because a disruption of the MEK4 gene results in an embryonic lethal phenotype, indicating that MEK7 cannot compensate for its absence either because of functional or expression differences (63). Thus, there may be distinct types of stress kinase-MEKK1 complexes.

MEKK1 binds the enzymes from two MAP kinase modules; nevertheless, the interactions appear to be specific. No association is detected either in vitro or in cells with the MAP kinases p38 and ERK3, the p38-selective MEK TAO2, and the other protein kinases PAK1 and WNK1, under conditions in vitro or in cells in which the ERK1/2 and JNK/SAPK module kinases are clearly associated. These results are consistent with the minimal effect of overexpressed MEKK1 on p38 and ERK5 activation (32, 64). The binding of Raf-1 to MEKK1 suggests that activation of ERK1/2 by MEKK1 may involve Raf-1. This would be consistent with the characterization of the specificity of endogenous MEKK1, which phosphorylated MEK4 but not MEK1 or 2, in contrast to recombiant enzyme that phosphorylated both (29, 42, 45).

Pbs2p is a yeast MEK family member and a scaffold (25). The MEKK it binds is Ste11p. When bound to Ste5p, Ste11p activates the MEK and MAP kinase involved in the mating pathway, but when bound to Pbs2p, it selectively mediates an osmotic response by activating a distinct MAP kinase. How Ste11p is directed to a particular scaffold is unclear; however, the fact that Ste5p is expressed primarily or exclusively in haploid cells may be a significant determinant (65). One function of the binding of Ste11p to these scaffolds is almost certainly to prevent it from phosphorylating MEKs not in the complex. Thus, formation of a complex creates a mechanism to restrict its intracellular specificity (66–68).

Perhaps the formation of complexes on MEKK1 will restrict the specificity of MEKK1 and the functions of the enzymes so associated. Studies in fruit flies, nematodes, and mammals suggest that kinase suppressor of Ras may enhance signaling to the MAP kinase module by Ras (16–18). Kinase suppressor of Ras complexes may be formed primarily to mediate Ras-dependent signaling from the plasma membrane. MEKK1, on the other hand, is tightly associated with the cytoskeleton (32, 33, 35, 60). Thus, one function of the complex between MEKK1 and the ERK2 MAP kinase module may be to sequester a population of Raf-1, MEK1, and ERK2 to the cytoskeleton to allow for efficient responses to signals that are transmitted through cytoskeletal elements. It is possible that the Raf-1 associated with MEKK1 is functionally distinct from pools of Raf-1 that respond to other activators such as Ras, protein kinase C, or Src. The same may also be true of the population of JNK/SAPK pathway components bound to MEKK1. JNK/SAPK also binds to the JIP proteins that may dictate their activation by specific regulators, distinct from those that communicate with MEKK1, and localize their actions to other sites in cells (69). Binding of MAP kinase modules to proteins such as MEKK1 may provide the cell with signaling specificity by organizing generic downstream components for regulation by a subset of possible activators to control a fraction of the possible functions. Overexpression of MEKK1 apparently overwhelms this sort of specificity determinant and instead reveals a large and unsorted array of biological activities stimulated outside of the appropriate biological context.

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