MEK Kinase 3 Directly Activates MKK6 and MKK7, Specific Activators of the p38 and c-Jun NH2-terminal Kinases*

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Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase 3 (MEKK3) activates the c-Jun NH2-terminal kinase (JNK) pathway, although no substrates for MEKK3 have been identified. We have examined the regulation by MEKK3 of MAPK kinase 7 (MKK7) and MKK6, two novel MAPK kinases specific for JNK and p38, respectively. Coexpression of MKK7 with MEKK3 in COS-7 cells enhanced MKK7 autophosphorylation and its ability to activate recombinant JNK1 in vitro. MKK6 autophosphorylation and in vitro activation of p38α were also observed following coexpression of MKK6 with MEKK3. MEKK2, a closely related homologue of MEKK3, also activated MKK7 and MKK6 in COS-7 cells. Importantly, immunoprecipitates of either MEKK3 or MEKK2 directly activated recombinant MKK7 and MKK6 in vitro. These data identify MEKK3 as a MAPK kinase kinase specific for MKK7 and MKK6 in the JNK and p38 pathways. We have also examined whether MEKK3 or MEKK2 activates p38 in intact cells using MAPK-activated protein kinase-2 (MAPKAPK2) as an affinity ligand and substrate. Anisomycin, sorbitol, or the expression of MEKK3 in HEK293 cells enhanced MAPKAPK2 phosphorylation, whereas MEKK2 was less effective. Furthermore, MAPKAPK2 phosphorylation induced by MEKK3 or cellular stress was abolished by the p38 inhibitor SB-203580, suggesting that MEKK3 is coupled to p38 activation in intact cells.

Multiple mitogen-activated protein kinase (MAPK) signaling pathways have been identified in mammalian cells that are activated in response to extracellular stimuli and cellular stress (for reviews, see Refs. 1–4). MAPK family members include the extracellular signal-regulated kinases (ERKs) (1, 2), the c-Jun NH2-terminal kinases (JNKs) (5, 6), and the p38 kinases (7–12). ERKs are activated by agonists for tyrosine kinase-encoded receptors and G protein-coupled receptors that induce mitogenesis, differentiation, or hypertrophy (1, 2), whereas the JNK and p38 subgroups are activated by cellular stress (e.g. UV and γ-irradiation, osmotic stress, heat shock, protein synthesis inhibitors) and inflammatory cytokines (e.g. TNF-α and interleukin-1) (3–9, 11, 12). The stress-activated kinases have been implicated in apoptosis (3–15), oncogenic transformation (16–18), and inflammatory responses (19, 20) in various cell types.

MAPK activation requires dual phosphorylation on Thr and Tyr within the motif Thr-Xaa-Tyr, where Xaa represents Glu in ERK, Pro in JNK, and Gly in p38 (2, 9). Specific MAPK kinases have been identified for each MAPK subgroup that allow for their selective activation (2, 3). Thus MEK1 and MEK2 selectively phosphorylate and activate the ERK subgroup (21, 22), whereas MKK5 and MKK6 selectively activate p38 (9, 11, 12, 22–25). MKK4 (also known as stress-activated protein kinase/ERK kinase 1 or JNK kinase) does not activate the ERK subgroup but activates both p38 and JNK (9, 22, 25–27). A second JNK kinase, designated MKK7, has recently been identified as a specific activator of the JNK subgroup (28–31).

In turn, activation of MAPK kinases involves dual phosphorylation on Thr and Ser residues within a conserved sequence located between kinase subdomains VII and VIII (22, 32–35). Raf-1 is a MAPK kinase (MAPKKK) that activates ERK via direct MEK phosphorylation at these sites (32–34). Raf-1 activation by cell surface receptors involves Ras-GTP, which binds to the NH2-terminal domain of Raf-1, causing its translocation and activation at the plasma membrane (1, 2, 32). Other Raf isoforms include A-Raf and B-Raf, and these also activate the MEK/ERK cascade (2, 32). Indeed, evidence to date suggests that Raf kinases represent the primary activators of the MEK/ERK pathway in vivo.

Additional MAPKKKs exist in mammalian cells that differ structurally from Raf and preferentially regulate the stress-activated protein kinase pathways (3, 4). Members of the MEKK family of serine/threonine kinases were the first MAPKKKs shown to activate JNK in cells (36) and directly phosphorylate MKK4 in vitro (27, 35, 37–39). Other MAPKKKs that directly activate MKK4 in the JNK cascade include tumor progression locus-2 (40); transforming growth factor-β-activated kinase-1 (TAK-1) (41); the mixed lineage kinases MLK-2 (42), MLK-3 (43), and MAPK upstream kinase (42); MAPK kinase kinase-5 (MAPKKK5) (44); apoptosis signal-regulating kinase-1 (ASK1) (45); and a human MEKK homologue termed MAP three kinase-1 (46). Additionally, ASK1 (45), MAPK three kinase-1 (46), and TAK1 (24) have been demonstrated to activate p38 and to directly phosphorylate MKK3 and/or MKK6 in vitro. Thus, there exists a tremendous diversity in the potential pathways by which MEKKs and related MAPKKKs regulate the stress-activated protein kinases in a stimulus and cell type-specific manner.

Four mammalian MEKK isoforms have been identified by virtue of their sequence homology with Ste11 and Byr2 (38, 39, 47), protein kinases involved in the pheromone mating re...
sponse pathways in yeast. The cDNAs for MEKK1 and -4 encode proteins of 161 (47) and 180 kDa (39), whereas those for MEKK2 and -3 predict proteins of 70 and 71 kDa, respectively (38). MEKK2 and -3 are closely related in primary sequence, sharing 77% identity overall and 94% identity between their carboxyl-terminal catalytic domains (38). MEKK2 and -3 are less closely related to other MEKKs, showing approximately 50% identity to MEKK1 and -4 through their respective catalytic domains (39). Recent work has established that MEKK1, -2, and -4 can activate JNK and directly phosphorylate and activate MKK4 (27, 35, 37–39), whereas MEKK3 strongly induces JNK activity in cells but is inactive with MKK4 in vitro or, indeed, with any other substrate so far examined (MEK1, MEK2, MEKK3) (35, 38). These observations suggest that MEKK3 may mediate JNK activation via a novel MAPK kinase distinct from MKK4. In the present study we have examined the ability of MEKK3 to regulate MKK7 (28–31) and MKK6 (23–25), two recently identified MAPK kinases specific for the JNK and p38 subgroups. We show that MEKK3 is a MAPKKK that preferentially activates these novel stress-activated protein kinase cascade.

**EXPERIMENTAL PROCEDURES**

*Plasmids—* Constructs for bacterial expression of MKK3, MKK4, MKK6, MKK7, JNK1, p38α, ATFP2, and c-Jun as translational fusions with glutathione-S-transferase (GST) were kind gifts from Dr. Roger J. Davis and have been described previously (22, 23, 25). The bacterial expression vector encoding amino acids 46–400 of MAPKAPK2 and fused to GST (48) was generously provided by Prof. Christopher J. Marshall. The GST fusion proteins were purified by affinity chromatography (49) on glutathione-Sepharose (Amersham Pharmacia Biotech). Purified fusion proteins were resolved by SDS-polyacrylamide gel electrophoresis using 10 or 12% polyacrylamide in the presence of 0.1% SDS and visualized by Coomassie Blue staining using bovine serum albumin to construct a standard curve. The Flag epitope tag DYKDDDDK was introduced between codons 1 and 2 of MEKK2 and MEKK3 by insertional overlapping polymerase chain reaction and cloned into the poly linker of pCMV5 (51) for mammalian expression as described (35). Flag-tagged MKK6 and MKK7 were similarly prepared (23, 28) and expressed from pCDNA3 (Invitrogen).

*Cell Culture and Transfection—* COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium at 37 °C in a 5% CO2, 95% air mixture. HEK293 cells were maintained under similar conditions in minimum essential medium (Life Technologies, Inc.). Media were supplemented with 1% fetal bovine serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (50). COS-7 cells were transfected with plasmid DNA as described (35). Rabbit polyclonal antisera selective for ERK1 and JNK1 by Flag-MKK6 and Flag-MKK7, respectively. Western blot analysis of Flag-MKK and MEKK expression using anti-Flag M2 and B161 antisera.

*Vector Expression—* COS-7 cells were transfected with empty vector (pCMV5, lanes 1 and 5), Flag-MKK6 (lanes 2–4), or Flag-MKK7 (lanes 6–8) in the presence of empty vector (pCMV5, lanes 2 and 6), MKK3 (lanes 3 and 7), or MEKK2 (lanes 4 and 8). Flg-MKK6 and Flag-MKK7 were isolated from lysates by immunoprecipitation, and their protein kinase activities were assessed in vitro as described under “Experimental Procedures.” A, effect of MEKK3 or MEKK2 on Flag-MKK6 and Flag-MKK7 autophosphorylation. B, effect of MEKK3 or MEKK2 on phosphorylation of GST-p38α and GST-JNK1 by Flag-MKK6 and Flag-MKK7, respectively. C, Western blot analysis of Flag-MKK and MEKK expression using anti-Flag M2 and B161 antiserum.

**RESULTS**

**Activation of MKK6 and MKK7 by MEKK3 and MEKK2** in COS-7 Cells—Flag epitope-tagged MKK6 and MKK7 were separately expressed in COS-7 cells either alone or in combination with MEKK3 or MEKK2. The MKKs were then isolated by immunoprecipitation using a monoclonal antisera to the Flag epitope and their protein kinase activities assessed in vitro. Fig. 1A shows that MKK6 autophosphorylation was low relative to that of MKK7. However, the autokatelic activities of...
MEKK3 Directly Activates MKK6 and MKK7

![Diagram](image-url)

Fig. 2. MEKK3 and MEKK2 can activate p38α and JNK1 via MKK6 and MKK7. A and B, COS-7 cells were transfected with empty vector (pCMV5, lanes 1, 2, 9, and 10), Flag-MKK6 (lanes 3–8), or Flag-MEKK7 (lanes 11–16) in the presence of empty vector (pCMV5, lanes 3, 4, 11, and 12), MEKK3 (lanes 5, 6, 13, and 14), or MEKK2 (lanes 7, 8, 15, and 16). Flag-MKK6 and Flag-MEKK7 were isolated by immunoprecipitation and assayed using either GST-p38α (A, lanes 1, 3, 5, and 7) or GST-JNK1 (B, lanes 9, 11, 13, and 15) as in vitro substrates. Activation of GST-p38α and GST-JNK1 was assessed using GST-ATF2 (A) and GST-c-Jun (B) as substrates.

Both MKK6 and MKK7 were enhanced by their coexpression with either MEKK3 or MEKK2. MKK6 and MKK7 activity was directly assessed in the immune complex kinase assay using purified recombinant p38α or JNK1 as substrates, respectively. These data confirmed that the activities of MKK6 and MKK7 were enhanced by their coexpression with either MEKK3 or MEKK2, although p38α appeared to act as a better substrate for MKK6 than did JNK1 for MKK7 (Fig. 1B). Western blot analysis using the anti-Flag antibody showed that expression of MKK6 or MKK7 was essentially unaffected by MEKK coexpression (Fig. 1C). An antiserum (B161), which at low dilution recognizes both MKK2 and MEKK3, confirmed expression of these kinases in the appropriate cell lysates (Fig. 1C).

To determine whether MKK-catalyzed phosphorylation of recombinant JNK1 and p38α resulted in activation of these MAPKs, immune complex kinase assays of Flag-tagged MKK6 and MKK7 were performed in which the transcription factors ATF2 or c-Jun were provided as in vitro substrates for p38α or JNK1, respectively. Fig. 2A shows that ATF2 phosphorylation by p38α was stimulated by immunoprecipitates of MKK6 obtained from cells coexpressing either MEKK3 or MEKK2. Similar data were obtained in parallel experiments in which JNK1 and c-Jun were used as the sequential substrates of MKK7 (Fig. 2B). Omission of either p38α (Fig. 2A) or JNK1 (Fig. 2B) from the in vitro reactions showed that the activated MKKs did not directly phosphorylate ATF2 or c-Jun, thereby confirming

the specificity of the assays. Thus, MEKK2 and MEKK3 are both capable of activating MKK6 and MKK7 in COS-7 cells.

MEKK3 Directly Activates MKK6 and MKK7 in Vitro—Previous studies have failed to identify any direct substrates of MEKK (35, 38), although cotransfection experiments have shown that MKK3 and MKK4 can be activated by MEKK3 in COS-7 cells (35). Because MEKK3 also activates MKK6 and MKK7 in intact cells (Fig. 2), we determined whether MEKK3 was capable of directly activating MKK6 or MKK7 in vitro. Flag epitope-tagged MEKK3 was expressed in COS-7 cells and isolated by immunoprecipitation. MEKK3 activity was then assessed in the immune complex kinase assay using recombinant MKK3, MKK4, MKK6, or MKK7 as substrates. To fully reconstitute each activation pathway, recombinant JNK1 and c-Jun were provided with MKK4 or MKK7, whereas recombinant p38α and ATF2 were provided with MKK3 or MKK6. Consistent with previous studies (35), immunoprecipitated MEKK3 failed to activate MKK3 or MKK4 in vitro (Fig. 3A). By contrast, immunoprecipitates of MEKK3 directly activated MKK6 and MKK7, resulting in a large increase in phosphorylation of the relevant transcription factor by p38α and JNK1, respectively (Fig. 3A). Immunoblot analysis showed equivalent expression of the Flag-MEKK2 protein used for the reconstitution assays (Fig. 3B). Thus, MEKK3 is a MAPK kinase kinase that shows selectivity in its activation of MKK6 and MKK7 over MKK3 or MKK4 in vitro.

Immunoprecipitates of Flag-tagged MEKK2 from COS-7 cells strongly autophosphorylated and also directly activated MKK6 and MKK7 when the kinase cascades were reconstituted using the relevant MAPKs and transcription factors in

![Diagram](image-url)

Fig. 3. MEKK3 directly activates MKK6 and MKK7 in vitro but not MKK3 or MKK4. COS-7 cells were transfected with either empty vector (pCMV5, lanes 1, 3, 5, and 7) or Flag-MEKK3 (lanes 2, 4, 6, and 8). A, Flag-MEKK3 was isolated by immunoprecipitation and assayed using GST-MKK3 (lane 2), GST-MKK4 (lane 4), GST-MKK6 (lane 6), or GST-MKK7 (lane 8) as in vitro substrates. Activation of GST-MKK3 and GST-MKK7 was assessed using GST-p38α and GST-ATF2 as sequential substrates (lanes 1, 2, 5, and 6), whereas GST-MKK4 and GST-MKK7 activity was assessed using GST-JNK1 and GST-c-Jun as substrates (lanes 3, 4, 7, and 8). B, Flag-MEKK3 expression was detected by immunoblotting with anti-Flag M2 antibody.
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A

Flag-MEKK2

Flag-MEKK2

Flag-MEKK2

GST-MKK7

GST-MKK6

GST-c-Jun

GST-p38

GST-ATF2

1 2 3 4 5 6 7 8 9 10

FIG. 4. MEKK2 directly activates MKK6 and MKK7 in vitro. COS-7 cells were transfected with either empty vector (pCMV5, lanes 1 and 6) or Flag-MEKK2 (lanes 2–5 and 7–10). A, Flag-MEKK2 was isolated by immunoprecipitation and assayed using GST-MKK7 (lanes 2 and 5) or GST-MKK6 (lanes 7 and 10) as in vitro substrates. Activation of GST-MKK7 was assessed using GST-JNK1 and GST-c-Jun as sequential substrates (lane 2), whereas GST-MKK6 activity was assessed using GST-p38α and GST-ATF2 as substrates (lane 7). Control assays were performed in which the MKK (lanes 3, 4, 8, and 9) and/or the MAPK (lanes 4, 5, 9, and 10) was omitted from the in vitro reaction. B, Flag-MEKK2 expression was detected by immunoblotting with anti-Flag M2 antibody.

B

Anti-Flag M2

Flag-MEKK2

Flag-MEKK2

1 2 3 4 5 6 7 8

FIG. 5. MKK7 and MKK6 are specific for JNK1 and p38α following activation by MEKK3. COS-7 cells were transfected with either empty vector (pCMV5, lanes 1, 3, 5, and 7) or Flag-MEKK3 (lanes 2, 4, 6, and 8). Flag-MEKK3 was isolated by immunoprecipitation and combined with either GST-MKK7 (lanes 2 and 4) or GST-MKK6 (lanes 6 and 8) in vitro. The ability of activated GST-MKK7 and GST-MKK6 to activate GST-JNK1 and GST-p38α was determined using GST-c-Jun and GST-ATF2 as substrates, as indicated.

MEKK3 is a Potential Activator of p38 in Intact Cells—MEKK2 and MEKK3 are established activators of the JNK pathway in vitro and in intact cells (35, 38). However, whereas p38α activation by MEKK2 and MEKK3 can be reconstituted using cognate MKKs in vitro (Figs. 1–5 and Ref. 35), previous studies have failed to demonstrate significant activation of p38α by its coexpression with either MEKK2 or MEKK3 in HEK293 cells (35). As MAPKAPK2 can serve as a substrate for the α, β, and γ isoforms of p38α (8, 10, 48), we have attempted to determine whether MEKK2 or MEKK3 is capable of stimulating a native p38 kinase activity in HEK293 cells using MAPKAPK2 as an affinity ligand to isolate p38 kinases and serve as their substrate (8, 10, 48, 52). Fig. 6A shows that MAPKAPK2 phosphorylation was enhanced following treatment of HEK293 cells with sorbitol or anisomycin, two stress activators of p38α, β, γ, and δ isoforms (7–12). Furthermore, SB-203580, a specific inhibitor of the p38 subgroup of MAPKs (8, 9, 19, 52), blocked this phosphorylation, thereby confirming that this assay detected p38 kinase activity. Additionally, expression of Flag-tagged MEKK2 or MEKK3 enhanced MAPKAPK2 phosphorylation and this could also be inhibited by SB-203580. Western blot analysis using anti-Flag monoclonal antiserum confirmed expression of MEKK3 and MEKK2 in the appropriate cell lysates (Fig. 6A). Immunoblot analysis also indicated that the use of GST-MAPKAPK2 as an affinity ligand allowed selective enrichment of p38α from HEK293 cell lysates, although some ERK binding could also be detected upon prolonged exposure of immunoblots (Fig. 6A). Thus, MEKK3 and MEKK2 may activate a native p38 kinase in HEK293 cells. Of note, MEKK3 reproducibly induced MAPKAPK2 phosphorylation, whereas MEKK2 was less effective.

To extend these observations, an antiserum raised to p38α was used to precipitate native p38 from cells that had been treated with sorbitol or anisomycin or transfected with Flag-tagged MEKK3 or MEKK2. p38 activity was measured in vitro using ATP2 as substrate. Although recovery of p38 kinase activity was low, detectable p38 activation could be observed by anisomycin, sorbitol, and MEKK3 (Fig. 6B). However, the potency of the effect did not correlate well with MAPKAPK2 phosphorylation, suggesting that MAPKAPK2 may precipitate additional isoforms of p38α that are not immunoprecipitated by the p38α-selective antiserum.

DISCUSSION

Recent molecular cloning studies have revealed the existence of numerous MAPKKKS in mammalian cells (1–4, 32, 38–47). It seems likely that this diversity provides multiple mechanisms for activation of MAPK subgroups in a stimulus- and cell type-specific manner. In most cases, MAPK regulation by these MAPKKKS correlates with their ability to phosphorylate and activate MAPKKs selective for each subgroup of MAPK. Thus Raf-1 is a MAPKKK that directly phosphorylates and activates MEK1 and MEK2 in the ERK pathway (32–34), whereas the
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JNK activator, MKK4, does not serve as a Raf-1 substrate (27). Conversely, MEKK isoforms can function as MAPKKKs in the ERK and JNK signaling pathways (27, 33, 35–39). Several studies indicate that MEKK1 may activate MKK4 and/or JNK in preference to ERK (36, 37, 47), whereas MEKK2 and MEKK3 strongly activate both the JNK and ERK pathways (38). MEKK4 shows the greatest selectivity for the MKK4/JNK cascade and does not appear to activate MEK or ERK (39). Other MAPKKKs that activate JNK have also been demonstrated to directly phosphorylate and activate MKK4 in vitro, including tumor progression locus-2 (40), TAK-1 (41), MLK-2 (42), MLK-3 (43), MAPK upstream kinase (42), MAPKAPK5 (44), ASK1 (45), and the human homologue of MEKK4 termed MAPKAPK3 (46). However, JNK activation by MEKK3 appears atypical in that it does not appear to involve MKK4 (35, 38). Indeed, despite its close homology to MEKK2, a direct activator of MEK1, MEK2, and MKK4, previous studies have failed to identify any MAPKK as a substrate for MEKK3 (35, 38).

Recent work has identified MKK7 as a specific activator of JNK (28–31). This selectivity differs from that of MKK4, which is capable of activating the JNK and p38 subgroups (8, 22, 25–27). In the present study, we have addressed whether MEKK3 and its close structural homologue, MEKK2, are capable of regulating MKK7. Our data show that MEKK3 and MEKK2 are both capable of activating MKK7 in intact cells and in vitro. Thus, MEKK3 represents a MAPKKK that selectively activates MKK7 in the JNK pathway, whereas MEKK2 is also capable of activating MKK4. Very recently, MEKK1 and MLK2 have also been shown to directly activate MKK7 (53). Interestingly, whereas MEKK1 activates MKK7 and MKK4 to similar extents, MLK2 appears to be a more effective activator of MKK7 than MKK4 (53). Thus, the selectivity of MEKK3 appears to be similar to that of MLK2, except that MEKK3 shows an absolute specificity for MKK7 over MKK4. As has been postulated for MLK2 (53), the specificity shown by MEKK3 may enable selective activation of MKK7 by extracellular stimuli and cellular stress. The observation of MKK7 activation by TNF-α in the absence of detectable MKK4 stimulation (31) suggests that MEKK3 may be involved in the preferential activation of MKK7 initiated by such a stimulus. Of note the JNK-interacting protein, JIP, is capable of binding JNK, MKK7, and MEKK3 but not MKK4 (54). The proposed role of JIP as a molecular scaffold (54) is consistent with the substrate selectivity of MEKK3 for MKK7 and may facilitate JNK signaling via this cascade.

Certain MAPKKKs have been identified as components of p38 signaling pathways. Those shown to activate p38 and directly phosphorylate and activate MKK3 and/or MKK6 include TAK1 (24) and ASK1 (45). Most studies indicate that MEKK types 1–4 do not activate p38 (27, 35, 38, 39), although MEKK1 (47, 55) and MAPK three kinase-1 (human MEKK4) (46) may cause modest activation of p38 cascades. Previous studies from this laboratory indicate that neither MEKK2 nor MEKK3 is capable of directly activating MKK3 in vitro (35), consistent with their lack of effect on p38 kinase activity in intact cells. It is surprising, therefore, that MEKK3 is capable of activating MKK3 when their corresponding cDNAs are transiently coexpressed in COS-7 or HEK293 cells. Activation of MKK3 requires its phosphorylation on Ser-189 and Thr-193 and is specific for MEKK3 in that MEKK2 is without effect in parallel experiments (35). However, the failure of MEKK3 to activate MKK3 in vitro suggests that the effects of MEKK3 on MKK3 activity in cells may be indirect.

To further define the function of MEKK3 and MEKK2 in p38 signaling, we have examined the regulation of MKK6 by these MEK kinases. We show that MEKK3 and MEKK2 can activate MKK6 in intact cells and in vitro. Thus, MEKK3 and MEKK2 are candidate MAPKKKs for the MKK6/p38 signaling pathway. Based on the apparent specificity of MEKK3 and MEKK2 for MKK6 as a target substrate, we have re-examined the potential for p38 regulation by both MEKKs. Four principle isoforms of p38 have been identified and are designated α (7), β (8, 9), γ (10), and δ (11, 12). Interestingly, recent evidence suggests that individual p38 subtypes may be differentially activated by MKK3 and MKK6 (8, 9, 11). Other work has demonstrated that p38α can phosphorylate MAPKAPK2 at Thr-222, Ser-272, and Thr-344 in vitro, resulting in activation of the kinase (48). In addition, activation of MAPKAPK2 in cells can be blocked by SB-203580 (48), a pyridyl imidazole compound that inhibits p38 kinase activity in cells (19, 52) and directly inhibits p38 α, β1, and β2 isoforms in vitro (8–10). MAPKAPK3 is also activated by p38 in vitro and in vivo and can associate directly with recombinant and endogenous forms of p38 (56). Thus, we have examined the ability of MEKK3 and MEKK2 to activate native p38 in HEK293 cells using MAP-
KAPK2 as an affinity ligand and a substrate. Our data indicate that GST-MAPKAPK2 can be used to selectively isolate endogenous p38 from cells, as demonstrated for GST-MAPKAPK3 previously (56). Furthermore MAPKAPK2 served as an in vitro substrate for associated kinase(s) that were activated by sorbitol, anisomycin, MEKK3, and MEKK2 to a lesser extent. Importantly, this MAPKAPK2 phosphorylation was blocked by the p38 inhibitor SB-203580. Thus, these studies suggest that MEKK3 and MEKK2 are coupled to MKK6 and p38 activation in vivo. As SB-203580 does not inhibit ERK, JNK (19), p38 (9, 10), or p38 (12), the endogenous p38 activity induced by MEKK3 and MEKK2 may include the p38α and/or p38β subtypes. Although immunoprecipitates of native p38 could be activated by MEKK3 and MEKK2, these effects were modest and have not been apparent by coexpression of either MEKK with recombinant p38α (35). Clearly, identification of the p38 isoform(s) regulated by MEKKs merits further investigation.

Although the intracellular mechanisms involved in MEKK regulation remain largely unknown, the noncatalytic NH2-terminal domains of MEKK1–4 are highly divergent, suggesting unique regulatory roles for each. For example, this domain in MEKK1 allows for its association with the particulate fraction and two proline-rich regions, a cysteine-rich region, and two potential pleckstrin homology domains (47). The NH2-terminal region of MEKK1 is inhibitory as caspase-dependent cleavage at the DTVQ sequence located between amino acids 870 and 875 in MEKK1 activates the enzyme (57). This mechanism may account for MEKK1-mediated JNK activation and anokis when Madin-Darby canine kidney cells lose contact with the extracellular matrix (57). The NH2-terminal region of MEKK4 has low homology to that of MEKK1 but shares a d10), or p38

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