Molecular Cloning of Mitogen-activated Protein/ERK Kinase Kinases (MEKK) 2 and 3

REGULATION OF SEQUENTIAL PHOSPHORYLATION PATHWAYS INVOLVING MITOGEN-ACTIVATED PROTEIN KINASE AND c-Jun KINASE*

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Mitogen-activated protein/ERK kinase kinases (MEKKs) phosphorylate and activate protein kinases which in turn phosphorylate and activate the p42/44 mitogen-activated protein kinase (MAPK), c-Jun stress-activated protein kinases (J Nks), and p38/Hog1 kinase. We have isolated the cDNAs for two novel mammalian MEKKs (MEKK 2 and 3). MEKK 2 and 3 encode proteins of 69.7 and 71 kDa, respectively. The kinase domains encoded in the COOH-terminal moiety of 94% are conserved; the NH2-terminal moieties are approximately 65% homologous, suggesting this region may encode sequences conferring differential regulation of the two kinases. Expression of MEKK 2 or 3 in HEK-293 cells results in activation of p42/44MAPK and JNK but not of p38/Hog1 kinase. Immunoprecipitated MEKK 2 phosphorylated the MAP kinase kinases, MEK 1, and J NK kinase. Titration of MEKK 2 and 3 expression in transfection assays indicated that MEKK 2 preferentially activated JNK while MEKK 3 preferentially activated p42/44MAPK. These findings define a family of MEKK proteins capable of regulating sequential protein kinase pathways involving MAPK members.

A variety of extracellular signals including growth factors, hormones, cytokines, antigens, and stresses such as heat shock and osmotic imbalance activate members of the mitogen-activated protein kinase (MAPK)1 family (1–5). MAPKs are characterized as serine/threonine-protein kinases activated by dual phosphorylation on both a tyrosine and a threonine (6). The MAPK family includes p42/44MAPK (also referred to as ERK2 and -1) (7), the c-j un kinases (J Nks which are also referred to as stress-activated protein kinases) (8), and p38, the osmotic imbalance responsive kinase similar to the yeast Hog1 enzyme (5). The regulation of different MAPKs including p42/44MAPK, J Nks, and p38 involves sequential protein kinase pathways whose upstream activators include the MEKs (MAPK/ERK kinases) and the J NK kinases (also referred to as SEKs or stress/ERK kinases) (9–13). MEKs and J NK kinases (J Nks) phosphorylate specific MAPK family members on both a tyrosine and threonine resulting in MAPK activation.

Raf-1 and B-Raf are serine/threonine-protein kinases that selectively phosphorylate and activate MEK 1 and MEK 2 (14–17). Recently, we isolated the cDNA for a novel serine/threonine-protein kinase referred to as MEK kinase (MEKK) 1 that phosphorylates and activates MEK 1 and 2 and J Nks (13, 18–20). The catalytic domain of MEKK 1 is homologous to the kinase domains of the Stel1 and Byr2 serine/threonine-protein kinases, involved in the control of mating in Saccharomyces cerevisiae and Schizosaccharomyces pombe, respectively (21–23). In this report, we have isolated and expressed the cDNAs for two new MEKK proteins, MEKK 2 and 3, when expressed in cells, are similar to MEKK 1 in that they are capable of regulating MEK and J NK activities. Thus, there exists a family of MEKKs controlling sequential protein kinase systems involving MAPK members in addition to the Raf family of protein kinases.

MATERIALS AND METHODS

Isolation of MEKK 2 and 3 cDNAs—The degenerate primers GA(G)/C(T)/T/A/G/T/I/G/G/T/I/A/G/A/C/G/T/A/G/A/G/T/G (antisense) and TT/G/G/T/I/G/T/T/G/T/I/G/G/C/G/T/A/G/A/G/T/G (sense) were used in a polymerase chain reaction (PCR) using first strand cDNA generated from polyadenylated RNA prepared from NIH 3T3 cells. The PCR reaction involved 30 cycles (1 min, 94 °C, 2 min, 52 °C, 3 min, 72 °C) followed by a 10-min cycle at 72 °C. A band of approximately 300 base pairs was recovered from the PCR mixture, and the products were cloned into pGEM-T (Promega).

The PCR cDNA products were sequenced and compared to the MEKK 1 sequence (19). A unique cDNA sequence of 328 base pairs having significant homology to MEKK 1 cDNA was identified and used to screen a dIg(dT)-primed mouse brain cDNA library (Stratagene). The λ phage library was plated, and DNA from plaques were transferred to Hybond N filters (Amersham) followed by UV-cross-linking of DNA to the filters. Filters were prehybridized for 2 h and then hybridized overnight in 0.5 Na2H2PO4 (pH 7.2), 10% bovine serum albumin, 1 mM EDTA, 7% SDS at 68 °C. Filters were washed twice at 42 °C with 2 × SSC, once with 1 × SSC, and once with 0.5 × SSC containing 0.1% SDS (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Positive hybridizing clones were purified and sequenced. To resolve GC-rich liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid.
regions, CDNAS were subcloned into M13 vectors (New England Bio-
labs), and single strand DNA was sequenced. In all cases, both strands of
the CDNAS were sequenced.

Plasmid Expression of MEKK 2 and 3—The proteins for MEKK 2 and 3 were
epitope-tagged at their NH2 termini with the hemagglutinin (HA)-tag sequence
GYPDYVPDYAS (24) using a PCR strategy as de-
scribed previously (11). For inserting the HA tag, a PCR fragment containing the
target sequence for MEKK 2 and 3, sense oligonucleotides were synthesized having a me-
thionine codon (ATG), 33 bases coding for the GYPDYVPDYAS epitope-
tag sequences, and 20 bases of MEKK 2 or 3 sequence starting at codon
2. For MEKK 2, the sense oligonucleotide was ATGGGATCCGGTA-
CGACGTGGGGATCCGTGTCGACGTTCACTTGAGATCACGACGT
TG GCCG AACTTGCTTGGAAGAAACAGGAGTATC. The anti-
sense oligonucleotides for MEKK 2 and 3 were AGA CTTA GATCTCAG
GTC TTC encoding a Bgl II site for MEKK 2 and GATTACGTCG
A C T C T G C C T G encoding an Aat II site for MEKK 3. The PCR reactions
were performed for 30 cycles using MEKK 2 or MEKK 3 cDNAs as template.
The PCR products were purified, and a second PCR reaction
was performed using the first PCR product as template, the MEKK 2 or MEKK 3 antisense oligonucleotide described above and the common sense oligonucleotide encoding a Xba I restriction site, a consensus Kozak initiation site and 17 bases overlapping with the initiation methionine, and a HA-tag sequence (TGACGTCTAGCAAGAAGGATCATACTCTGTCGTTCCC encoding a Bgl II site for MEKK 2 and GATTACGTCGACTCGTTGTTTTTG encoding an Aat II site for MEKK 3. The resulting PCR products were digested with Xba I and Bgl II and ligated into frame into the appropriate MEKK 2 or 3 CDNAS. The sequences were confirmed by DNA sequencing, and the CDNAS were inserted into the expression plasmid pcMVS. HEK293 cells were transfected with pcMVS expression plasmids using LipofectAMINE (Life Technologies, Inc.) and as-
sayed 48 h later. The 12CA5 monoclonal antibody (Berkeley Antibody Co.) was used for recognition of the HA epitope tag encoded in expres-
sed MEKK 2 and 3.

Antibody Production—Peptides corresponding to COOH-terminal se-
enes of MEKK 2 (CEARQRPSAEELLTHHFAQ) and p38 (CFVPP
PLDQEEMES) were conjugated to keyhole limpet hemocyanin and
used to immunize rabbits. Antibodies were characterized for specificity by
immunoblotting of lysates prepared from appropriately transfected
HEK293 cells.

Assay of JNK Activity—JNK activity was measured using GST (glu-
tathionine S-transferase)-c-Jun (1–79) coupled to glutathione-Sepharose 4B
(26). Cells transfected with MEKK 2 or 3 and control transfected cells
were lysed in 0.5% Nonidet P-40, 20 mM Tris (26). Cells transfected with Xba I and Bgl II and ligated in-frame into the appro-
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FIG. 1. DNA and deduced amino acid sequences for MEKK 2 and 3. A, MEKK 2; B, MEKK 3. In-frame stop codons 5' to the predicted start methionine are underlined.
quences for the differential regulation of the two proteins.

Fig. 2 shows the alignment of MEKK 1, 2, and 3 catalytic domains. The 11 conserved subdomains comprising the protein kinase catalytic domain are designated by Roman numerals. The COOH terminus of MEKK 1 encoding the catalytic domain is only 50% homologous to the corresponding region of MEKK 2 and 3. Thus, the catalytic domains of MEKK 2 and 3 are very similar to each other but significantly divergent from MEKK 1. As shown below, MEKK 1, 2, and 3 can all stimulate JNK and p42/p44MAPK activities in transfected cells. The significance of these sequence differences in the catalytic domains of MEKK 1, 2, and 3 is presently unclear.

MEKK 2 and 3 Activate c-Jun Kinase and p42/p44MAPK Activity—

Fig. 2, Comparison of amino acid sequences for MEKK 2 and 3. A, amino acids not having homology are boxed in the alignment of MEKK 2 and 3. B, alignment of the catalytic domains for MEKK 1, 2, and 3. Roman numerals indicate the 11 conserved regions within the protein kinase catalytic domain, with the most highly conserved residues underlined (32). Lowercase letters represent nonconserved amino acids in one or more of the MEKK sequences.
and 3 were expressed at similar levels in HEK293 cells when 2 μg of plasmid DNA was used per transfection (not shown). To determine whether MEKK 2 and 3 demonstrated selectivity in activating the JNK and p42/44MAPK pathways, plasmid DNAs were titrated over a range of concentrations in the transfections. Fig. 5 shows that MEKK 2 has a greater selectivity for stimulation of the JNK pathway. In contrast, MEKK 3 had a greater selectivity for activating p42/44MAPK relative to JNK. Thus, even though the kinase domains are approximately 94% conserved, MEKK 2 and 3 differ in their selectivity for regulation of the JNK and p42/44MAPK pathways. This was particularly evident for MEKK 3 at low plasmid concentrations where

the p42/44MAPK pathway was preferentially activated. MEKK 2 Phosphorylates Both MEK 1 and JNK Kinase in Vitro—HEK293 cells expressing MEKK 2 and 3 were lysed, and the recombinant MEK proteins were immunoprecipitated using the 12CA5 antibody recognizing the HA epitope tag. The immunoprecipitates were then used for in vitro kinase assays with recombinant purified MEK 1 and JNK kinase (JNKK) as substrates (Fig. 6A). MEKK 2 clearly phosphorylates both MEK 1 and JNKK consistent with its ability to activate JNK and p42/44MAPK in HEK293 cells. Fig. 6B shows that the MEKK 2-catalyzed phosphorylation of recombinant JNKK resulted in the enhancement of JNKK activity. Thus, JNKK is a MEKK 2 substrate whose activity is stimulated both in vitro and in vivo by MEKK 2. We were unable to demonstrate the ability of MEKK 3 to phosphorylate MEK 1 and MEK 2 (not shown), or JNKK in vitro using a variety of immunoprecipitation procedures. Although MEKK 3 was efficiently immunoprecipitated, as determined by Western blot analysis, it did not show measurable kinase activity toward MEK 1 or JNKK or show detectable autophosphorylation. This contrasted dramatically with the ability of MEKK 3 to activate both JNK and p42/44MAPK in cells (Figs. 3-5). MEKK 3 protein was clearly immunoprecipitated using the 12CA5 antibody in these experiments, and a rabbit antiserum raised against a keyhole limpet
activity of MEKK 1 or JNKK suggests one of three possibilities: (i) MEKK 3 immunoprecipitated MEKK 3 to phosphorylate recombinant protein indicating that it was not degraded. The failure of acids of MEKK 3 recognized the intact immunoprecipitated hemocyanin-conjugated peptide encoding the last 15 amino acids of MEKK 3 phosphorylated JNKK stimulates JNK kinase activity. MEKK 2 immunoprecipitates were incubated with the indicated combinations of wild-type or kinase-inactive JNKK and JNK as described under "Materials and Methods." GST-c-Jun (1–79) phosphorylation by JNK was used as a measure for activation of the JNKK/JNK pathway.

Phosphorylation of recombinant MEK 1 and J NKK by immunoprecipitated MEKK 2. A, HEK293 cells were transfected with pCMV5 alone (−) or encoding HA epitope-tagged MEKK 2 or 3. Forty-eight h post-transfection, cells were lysed, and MEKK 2 and 3 were immunoprecipitated using the 12CA5 monoclonal antibody. Immunoprecipitates were then assayed for kinase activity using recombinant kinase-inactive MEK 1 or J NKK as substrate. The J NKK only lane shows the low level of autophosphorylation of recombinant J NKK. Results are representative of 5–6 experiments for both MEKK 2 and 3. B, MEKK 2 phosphorylation of J NKK stimulates J NKK kinase activity. MEKK 2 immunoprecipitates were incubated with the indicated combinations of wild-type or kinase-inactive J NKK and J NKK as described under "Materials and Methods." GST-c-jun 1–79 phosphorylation by J NKK was used as a measure for activation of the J NKK/J NKK pathway.

hemocyanin-conjugated peptide encoding the last 15 amino acids of MEKK 3 recognized the intact immunoprecipitated protein indicating that it was not degraded. The failure of immunoprecipitated MEKK 3 to phosphorylate recombinant MEK 1 or J NKK suggests one of three possibilities: (i) MEKK 3 is denatured but not degraded during immunoprecipitation, (ii) MEKK 3 requires an additional protein or co-factor for its activity in vitro that is lost during immunoprecipitation, (iii) the relevant substrate for MEKK 3 in cells is neither MEK 1 or 2 nor J NKK. At present, it is not clear which of these possibilities is responsible for the failure to detect MEKK 3 activity in vitro. We demonstrated that a mutant MEKK 3 having lysine 391 mutated to methionine, rendering it kinase-inactive (19, 27, 32), did not stimulate J NKK or p42/44MAPK activity when expressed in HEK293 cells (not shown). This finding indicated that the functional kinase activity of MEKK 3 was required for the in vivo regulation of J NKK and p42/44MAPK.

MEKK 2 and 3 Do Not Regulate p38 Activity in HEK293 Cells—The p38 kinase is activated by hyperosmotic conditions (5) and recognizes the transcription factor ATF 2 as an in vitro substrate (33). Incubation of HEK293 cells with sorbitol activated p38 kinase (Fig. 7A). Immunoprecipitation and in vitro kinase assay of p38 from MEKK 2 and 3 transfected HEK293 cells indicated that neither MEKK 2 nor MEKK 3 stimulated p38 kinase activity (not shown). Mono Q FPLC fractionation of lysates from MEKK 2 or 3 transfected HEK293 cells confirmed that p38 kinase activity was similar to that from control transfected cells (Fig. 7B). ATF 2 is also a substrate for J NKK (31). Fractions 2–8 from cells transfected with MEKK 2 or 3, that contain immunoreactive J NKK, have increased kinase activity toward ATF 2. This is a predicted result based on the ability of both MEKK 2 and 3 to stimulate J NKK activity in HEK293 cells. Expression of MEKK 2 and 3 also activated additional ATF 2 phosphorylating activities resolved by Mono Q fractionation. These activities are seen to elute in fractions 9–12 and 13–18 for lysates from both MEKK 2 and 3 expressing cells. These activities do not correspond by immunoblotting to J NKK, p42/44MAPK, p38, or MEKK 2 or 3 and represent novel kinase activities capable of phosphorylating recombinant ATF 2 that are regulated by both MEKK 2 and 3.

FIG. 6. Phosphorylation of recombinant MEK 1 and J NKK by immunoprecipitated MEKK 2. A, HEK293 cells were transfected with pCMV5 alone (−) or encoding HA epitope-tagged MEKK 2 or 3. Forty-eight h post-transfection, cells were lysed, and MEKK 2 and 3 were immunoprecipitated using the 12CA5 monoclonal antibody. Immunoprecipitates were then assayed for kinase activity using recombinant kinase-inactive MEK 1 or J NKK as substrate. The J NKK only lane shows the low level of autophosphorylation of recombinant J NKK. Results are representative of 5–6 experiments for both MEKK 2 and 3. B, MEKK 2 phosphorylation of J NKK stimulates J NKK kinase activity. MEKK 2 immunoprecipitates were incubated with the indicated combinations of wild-type or kinase-inactive J NKK and J NKK as described under "Materials and Methods." GST-c-jun 1–79 phosphorylation by J NKK was used as a measure for activation of the J NKK/J NKK pathway.

B

| MEKK 2 | + | + | + | + | + |
|-------------|---|---|---|---|---|
| wt JNKK     | + | - | + | - | + |
| kin" JNKK   | - | + | - | - | + |
| wt JNK      | + | + | + | + | + |
| kin" JNK    | - | + | - | - | + |

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Based on the biochemical characterization of MEKK proteins, it is evident that their activities are quite distinct from those of Raf-1 and B-Raf kinases. The Raf kinases selectively regulate MEK 1 and 2 and do not recognize the J NKK proteins (1, 14, 20, 39–35). Thus, Raf proteins which evolved in metazoan organisms appear to be highly selective for the regulation of p42/44MAPK pathways (1, 8). At present, no distinct pathways or substrates other than MEK and p42/44MAPK have been defined for Raf kinases, although it is probable that additional MEK-like kinases will be identified in the future that serve as Raf substrates. MEKK proteins, in contrast, are capable of regulating both J NKK and p42/44MAPK pathways.

The ability of MEKKs to regulate multiple sequential protein kinase pathways in the cell suggests that a different mechanism exists for their regulation compared to the Raf kinases. The simplest prediction would be that MEKK proteins are selectively organized in "signalsome" complexes much like that for the mating pathway in S. cerevisiae. In this pathway, the protein kinases Ste11 (MEKK-like), Ste7 (MEK-like), and Fus3 (MAPK-like) are held together in a high affinity complex by Ste5 (36–38). Ste5 functions as a scaffold to keep these proteins in an organized complex. Expression of gain-of-function Ste11 mutants can result in overcoming a threshold where Ste11 and p42/44MAPK have been activated in vivo. The ability of Ste5 equivalent has yet been reported for mammalian cells and MEKKs. If such scaffold-like proteins do exist in mammalian cells and their expression is limiting, it would explain the ability of transiently expressed MEKKs to regulate both J NKK and p42/44MAPK pathways. It will obviously be necessary to define the organization of potential MEKK signalsome complexes in the cell and the constituent kinases in each complex.

DISCUSSION

The cloning and characterization of MEKK 2 and 3 define a family of MEKK proteins. MEKK 1, 2, and 3 are all capable of regulating both p42/44MAPK and J NKK activities. MEKK 1 and 2 appear to preferentially regulate the J NKK pathway, whereas MEKK 3 shows a preference for activation of the p42/44MAPK pathway in vivo. Cumulatively, our current and previous results (19) indicate that the different MEKKs when transiently expressed do not display a high selectivity for the p42/44MAPK or J NKK regulatory pathways. At more modest levels of expression, a greater degree of selectivity is observed for MEKK regulation of sequential protein kinase pathways. In MEKK 1-inducible clones of NIH3T3 (34) and Swiss 3T3 (35) cells, J NKK is preferentially activated relative to p42/44MAPK. MEKK 1, 2, and 3 do not measurably activate the p38 kinase pathway in these cell types.

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Finally, the MEKK/JNK pathways can be activated by a diverse set of stimuli. These include cytokines such as TNF and IL-1 (39), low molecular weight GTP-binding regulatory proteins including Ras, Rac, and Cdc42 (40, 41), high intracellular calcium (42), and stresses such as ultraviolet light, heat shock, osmotic imbalance, sphingomyelinase activity, protein synthesis inhibitors, etc. (1, 8, 13, 14, 20). Based on this array of stimuli capable of activating JNK, it is likely that several independent pathways converge to regulate the JNK sequential protein kinase pathway. It is possible that MEKK 1, 2, and 3 may all regulate the JNK pathway and each functions to respond to different upstream inputs. Alternatively, it is possible that MEKK 1, 2, and 3 are not only capable of regulating the JNK pathway but also other parallel sequential protein kinase pathways as well. Such a mechanism would allow coordinated regulation of both the JNK pathway and additional parallel sequential protein kinase pathways in the cell. In support of this hypothesis, we have found that MEKK 1 also selectively regulates a protein kinase pathway leading to the phosphorylation and transactivation of c-Myc that is independent of JNK and c-Jun (35). The magnitude of a specific MEKK activation in response to a stimulus could selectively regulate different pathways such as those for JNK and c-Myc kinase activity by requiring different thresholds of MEKK activity to be obtained for stimulation of each pathway. The thresholds for MEKK regulation of these pathways might be regulated in part by the relative abundance or cellular localization of specific signal-some complexes. The cloning of MEKK 2 and 3 allows us to now address these potential regulatory mechanisms for the three MEKK proteins using both genetic and biochemical approaches.

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FIG. 7. Measurement of p38 kinase activation in MEKK 2 and 3 transfected cells. A, stimulation of p38 kinase activity in response to sorbitol. HEK293 cells were incubated for 20 min with 0.4 M sorbitol. Cells were then lysed, and p38 was immunoprecipitated using a rabbit antiserum raised against a COOH-terminal peptide sequence of p38. Recombinant ATF 2 was used in an in vitro kinase assay as a substrate for p38 as described under “Materials and Methods.” The results are representative of two independent experiments. B, HEK293 cells were transfected with pCMV5 plasmids encoding no cDNA or MEKK 2 or 3. Lysates were prepared 48 h post-transfection and fractionated using a 0–0.5 M NaCl gradient on a Mono Q ion exchange column. Fractions were assayed for kinase activity using recombinant ATF 2 as substrate. JNK and p38 were identified in the column fractions by immunoblotting using specific antibodies for JNK and p38 (not shown).
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