Phosphorylation of Serine 526 Is Required for MEKK3 Activity, and Association with 14-3-3 Blocks Dephosphorylation*

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MAPK/ERK kinase 3 (MEKK3) is a mitogen-activated protein kinase kinase kinase (MAP3K) that functions upstream of the MAP kinases and IKK kinase. Phosphorylation is believed to be a critical component for MEKK3-dependent signal transduction, but little is known about the phosphorylation sites of this MAP3K. To address this question, point mutations were introduced in the activation loop (T-loop), substituting alanine for serine or threonine, and the mutants were transfected into HEK293 Epstein-Barr virus nuclear antigen cells. MEKK3-dependent activation of an NF-κB reporter gene as well as ERK, JNK, and p38 MAP kinases correlated with a requirement for serine at position 526. Constitutively active mutants of MEKK3, consisting of S526D and S526E, were capable of activating a NF-κB luciferase reporter gene as well as ERK and MEK, suggesting that a negative charge at Ser526 was necessary for MEKK3 activity and implicating Ser526 as a phosphorylation site. An antibody was developed that specifically recognized phospho-Ser526 of MEKK3 but did not recognize the S526A point mutant. The catalytically inactive (K391M) mutant of MEKK3 was not phosphorylated at Ser526, indicating that phosphorylation of Ser526 occurs via auto-phosphorylation. Endogenous MEKK3 was phosphorylated on Ser526 in response to osmotic stress. In addition, phosphorylation of Ser526 was required for MKK6 phosphorylation in vitro, whereas dephosphorylation of Ser526 was mediated by protein phosphatase 2A and sensitive to okadaic acid and sodium fluoride. Finally, the association between MEKK3 and 14-3-3 was dependent on Ser526 and prevented dephosphorylation of Ser526. In summary, Ser526 of MEKK3 is an autophosphorylation site within the T-loop that is regulated by PP2A and 14-3-3 proteins.

The mitogen-activated protein kinase (MAPK)8 pathways are divided into four separate groups known as MAPKERK, MAPKJNK, MAPKp38, and MAPKERKS (reviewed in Ref. 1). Within each group, there is a highly conserved three-kinase signaling module consisting of a MAPK, a MAPK kinase (MAP2K), and a MAPK kinase kinase (MAP3K). Regulation of the MAP3K, presumably by phosphorylation, provides the impetus for activation of the three-kinase module. Once activated, the MAP3Ks activate MAP2Ks by phosphorylation of two residues within the activation loop. Phosphorylation of MAP2Ks activates these dual specificity kinases to phosphorylate MAPKs on a conserved threonine and tyrosine motif, TXY, also within the activation loop. Once phosphorylated, the MAPKs phosphorylate protein substrates and regulate cellular processes like growth, protein synthesis, gene expression, and nucleotide synthesis (2).

Over the last decade, a large body of work has characterized events that occur downstream of the MAPKs. However, little is known regarding the regulatory mechanisms that modulate the MEKK proteins to ultimately regulate the MAPKs. For example, it is known that overexpression of MEKK3 activates the ERK (3, 4), JNK (3–5), p38 (5, 6), ERK5 (7), and NF-κB pathways (8–10). Typically, MEKK3-dependent regulation of these pathways is studied by using transfection studies, and activation of these pathways rarely requires an agonist. Therefore, it appears that some process intrinsic to MEKK3 is critical and sufficient for activation of these pathways.

The activation loop of some protein kinase families, such as the arginine-aspartate family, is positioned between subdomains VII and VIII and is phosphorylated by other protein kinases or through autophosphorylation of the kinase itself (11). Phosphorylation within the activation loop of protein kinases results in conformational changes in the protein structure that (i) enhance substrate binding, (ii) correctly position amino acids involved in catalysis, and (iii) relieve steric hindrance within the catalytic domain. Regardless of how the activation loop is phosphorylated, regulation of catalytic activity frequently correlates with phosphorylation of the activation loop.

Given that phosphorylation plays a critical role in regulating the MAPKs and the MAP2Ks, we investigated how phosphorylation of MEKK3 might affect its catalytic activity. Since phosphorylation sites within the activation loop of MEKK3 have not been reported, we systematically mutated serine and threonine residues within the activation loop to alanine and monitored MEKK3-dependent activities in HEK293 EBNA cells. Two key amino acids were identified at positions 526 and 530 using a luciferase-based reporter gene assay as well as assays that measure the ERK, JNK, and p38 MAP kinases. We demonstrate that Ser526 is phosphorylated by using a specific antibody that recognizes phospho-Ser526. In contrast, Thr530 is not phosphorylated but is required for MEKK3 catalytic activity. In addition, phosphorylation of endogenous MEKK3 occurs on Ser526 in response to osmotic stress. Finally, dephosphorylation of phospho-Ser526 is mediated by PP2A, and...
TABLE 1
Primer sequences used to generate point mutations in the MEKK3 activation loop

| Site   | Primer sequence | Endonuclease site |
|--------|-----------------|------------------|
| S511A  | TTT GGG GCC GCC AAA GCC CTG CAG ACC | PstI             |
| T516A  | CGC CTA CAG GCC ATC TGC ATG | Ndel            |
| S520A  | TGC ATG GCA GGG ACA GGC AAG ATT CTA TGG GTC ACT | PvuI |
| T522A  | TCA GGG GCA GGC ATT AGA TCT GTC ACT | BglII |
| S526A  | GCG ACA GGA ATT CCG TCT GTC ACT GGC | EcoRI |
| S526D  | TCA GGG ACC AAG ATT CCG GAT GTC ACT GGC | KpnI |
| S526E  | GGG ACA GGG ATT CCG GAA GTC ACT GGC | BamHI |
| T528A  | CGC TCT GTC GCC CCC GAA CAC CCC | Nael |
| T530A  | GTC ACT GGC GGC CCC TAC TGG | Nael |
| T530D  | GGC ATT AGA TCT GTC ACT GGC GAT CCC TAC | BglII |
| T530E  | GGC ATT CGA TCG GTC ACT GGC GAA CCC TAC TGG | PvuI |

the association of 14-3-3 with phosphorylated MEKK3 prevents dephosphorylation by PP2A.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies to phospho-ERK1/2, phospho-MEK, MEK1/2, and secondary antibodies conjugated to horseradish peroxidase were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against ERK1/2 and 14-3-3β were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The 14-3-3 antibody recognizes amino acids 3–21 of the human isoform of 14-3-3β, and this sequence is highly conserved within the 14-3-3 family of proteins (β, γ, ε, η, θ, and σ). An antibody to MEKK3 was generated as previously described (12). Monoclonal antibodies recognizing the hemagglutinin and the FLAG epitopes were obtained from Roche Applied Science. Anti-FLAG affinity resin was obtained from Sigma.

**Plasmids and Site-directed Mutagenesis**—FLAG-MEKK3 was excised from pBTM116 using Sall and ligated into the EcoRV site of pcDNA3.1 HisA (12). FLAG-MEKK3 in pcDNA3.1 HisA was used as a template for PCR to generate 370-bp fragments containing point mutations in the activation loop, replacing serine or threonine with alanine, aspartic, or glutamic acid. The 370-bp fragment was flanked by HindIII and NotI endonuclease sites. Since the 5′ Sall site was lost after ligation of FLAG-MEKK3 into pcDNA3.1 HisA, the NotI site of the polylinker was used as a 3′ cloning site in subsequent steps.

The megaprimer method of site-directed mutagenesis, which involved two PCR steps, was utilized to generate point mutants. Unique sense primers, listed in Table 1, and the antisense primer, 5′-T ACC TAG CAG GAA CAG ATT GTG TCG ACC TCA GTA CAC T-3′, were used in the first reaction (13). The product of the first reaction was used as a template for the second reaction with an MEKK3 sense primer, 5′-GCC ATG TCA TAC TCT CAC AGC-3′, which is upstream of the HindIII site, and antisense primer 5′-T ACC TAG CAT GAA CAG ATT G-3′. The product of this reaction was subcloned into pGEM-T and sequenced to verify the nucleotide sequence. The 370-bp HindIII site, and antisense primer 5′-T ACC TAG CAT GAA CAG ATT G-3′, were used as a template for the second reaction with an MEKK3 sense primer, 5′-GCC ATG TCA TAC TCT CAC AGC-3′, which is upstream of the HindIII site, and antisense primer 5′-T ACC TAG CAT GAA CAG ATT G-3′. The product of this reaction was subcloned into pGEM-T and sequenced to verify the nucleotide sequence. The 370-bp HindIII/NotI fragment containing the mutation was ligated with the 1800-bp KpnI/HindIII fragment of MEKK3 into pcDNA3.1 HisA that was cleaved with KpnI/NotI. The same procedure was used to construct the point mutants listed in Table 1.

**GST-lamin C** (amino acids 66–296) was prepared by digesting pLexA-Lamin, which was provided by Anne B. Vojtek (14), with EcoRI and Sall and ligating into pGEX-5X-1 that was digested with the same restriction enzyme.

**Cell Culture**—HEK293 EBNA cells were maintained at 37 °C, 5% CO2, with Dulbecco’s modified Eagle’s medium, pH 7.4 (Invitrogen), supplemented with 10% fetal bovine serum, 250 μg/ml Genetin, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Transfections**—HEK293 EBNA cells were transiently transfected with an empty vector as a control, MEKK3, K391M, or point mutants in the pcDNA3.1 HisA plasmid using 9 μl of Eugene 6 (Roche Applied Science) and 3 μg of DNA. Cells were transfected in OPTI-MEM serum-free medium (Invitrogen) and then replaced with complete medium 5 h post-transfection.

**Immunoprecipitation and Immunoblotting**—Cells were washed twice with ice-cold PBS and then lysed in buffer containing 20 mM Tris-HCl, pH 7.6, 0.5% Nonidet P-40, 0.25 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 0.25 μg/ml leupeptin, 1 μg/ml apro tinin. Cellular extracts were then cleared by centrifugation at 14,000 × g for 10 min at 4 °C. The protein concentration was determined using bovine serum albumin as a standard (15). For immunoprecipitations, 1 mg of cell extract was incubated with 10 μl of anti-FLAG affinity beads and rotated at 4 °C for 2 h. Samples were washed twice with 1 ml of PAN-Nonidet P-40 (10 mM Pipes, pH 7.0, 20 μg/ml aprotin, 100 mM NaCl (PAN), and 0.5% Nonidet P-40) and then with 1 ml of PAN. Immune complexes were collected by centrifugation at 2000 × g for 2 min, and the protein-bound beads were heated at 90 °C for 5 min in Laemmli sample buffer. Samples were resolved by 8% SDS-PAGE and probed with appropriate antibodies. For Western blots, 100 μg of cellular extract was resolved by 8% SDS-PAGE, transferred to nitrocellulose, and probed with appropriate antibodies.

**JNK Activity Assay**—This assay was performed as previously described (16). Briefly, cell extract (40 μg of protein) was incubated with 10 μg of GST-c-Jun bound to glutathione-Sepharose and rotated for 2 h at 4 °C. After washing with PAN and PAN-Nonidet P-40 as described above, precipitated JNK was subjected to an in vitro kinase assay with [γ-32P]ATP and then resolved by SDS-PAGE and visualized by autoradiography.

**NF-κB Luciferase Assay**—HEK293 EBNA cells were transiently co-transfected with 3 μg of MEKK3, K391M, or point mutants and 3 μg of NF-κB luciferase reporter construct (Promega, Madison, WI). Cellular extracts were prepared 48 h post-transfection using passive lysis buffer (Promega) and cleared by centrifugation at 14,000 × g for 10 min, and 10 μg of protein was used to measure luminescence with a TD luminometer (Turner Designs, Sunnyvale, CA).

**p38 MAP Kinase Assay**—HEK293 EBNA cells were transiently transfected as previously described above. Cell extracts were prepared by lysis in buffer containing 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 20 mM NaF, 0.2 mM Na3VO4, 1 mM EDTA, and 1 mM EGTA. The p38 MAP kinase was immunoprecipitated as described previously (17). Briefly, 1 mg of cellular extract was incubated with polyclonal anti-p38 antibody (provided by Gary Johnson) and then rotated at 4 °C for 1 h with Protein A conjugated to Sepharose (Sigma). Protein complexes were collected by centrifugation at 2000 × g.
Serine 526 Is Phosphorylated in the Activation Loop of MEKK3

for every 1 ml of gel. This reaction proceeded for 15 min with no mixing added to block nonspecific sites on the gel by applying 1 ml of cysteine supplemented with 1.5 mM EGTA, 0.5 mM DTT, and 0.12 mg/ml bovine serum albumin. The reactions were incubated in a volume of 20 μl at 30 °C, and after 1 h, 2× Laemmli sample buffer was added to the cell extracts, and the proteins were resolved by SDS-PAGE and then immunoblotted with anti-phosho-Ser526 antibody.

Phosphorylation of MKK6—FLAG-MEKK3 was transfected into HEK293 EBNA cells and immunoprecipitated as described above. Preparation of the cDNA that encodes GST-KA-MKK6 was previously described (19), and the GST portion of the fusion protein was removed by proteolysis with Factor Xa. The kinase assay was performed as follows in buffer containing 25 mM Tris-HCl, pH 7.4, 25 mM MgCl2, 1 mM EGTA, 2 mM DTT, 25 mM β-glycerophosphate, 50 μM ATP, 20 μCi of [γ-32P]ATP (1 Ci/mmol), and 1 μg of recombinant KA-MKK6, incubated at 30 °C for 20 min, and stopped by the addition of 40 μl of 2× Laemmli-DTT buffer. The proteins were denatured by heating for 5 min at 95 °C, resolved by 8% SDS-PAGE, and stained with Coomassie Blue, and the gel was dried prior to autoradiography.

Isolation of Proteins with GST-14-3-3ε—Cell extracts were prepared by lysis in buffer containing 20 mM Tris-HCl, pH 7.6, 0.5% Nonidet P-40, 0.25 M NaCl, 1 mM phenethylsulfonyl fluoride, 2 mM Na3VO4, 0.25 μg/ml leupeptin, 1 mM DTT, and 1 μg/ml aprotonin. Extracts were cleared of cellular debris by centrifugation at 14,000 × g for 5 min at 4 °C. Approximately 500 μg of cellular extract was incubated with 20 μg of recombinant GST-lamin (amino acids 66–206) or GST-14-3-3ε fusion proteins bound to Sepharose beads for 2 h at 4 °C. Samples were then washed twice with PAN-Nonidet P-40 and once with PAN, proteins were collected at 2000 × g of cellular extract containing endogenous protein phosphatases. The proteins were separated by SDS-PAGE, and after transfer to nitrocellulose, the membrane was probed with an amino-terminal antibody that recognizes total MEKK3 or phospho-Ser526, followed by goat anti-rabbit horseradish peroxidase antibody.

RESULTS

Ser526 and Thr530 Are Required for MEKK3-dependent NF-κB Luciferase Activity—MEKK3 has previously been shown to play a critical role in the NF-κB signal transduction pathway (9, 20), and therefore a NF-κB luciferase assay was chosen as one method to screen point mutants in the activation loop of MEKK3. To investigate which amino acids might be essential in the regulation of NF-κB-dependent transcription, each serine and threonine within the activation loop of MEKK3 was mutated to alanine by site-directed mutagenesis (Fig. 1A). Each point mutant of MEKK3 was transfected into HEK293 EBNA cells, along with a luciferase reporter gene containing the NF-κB recognition sequence. After 48 h, cell extracts were prepared and assayed for luciferase activity. Expression of MEKK3 resulted in agonist-independent transcription of the NF-κB luciferase reporter gene, whereas only the (S526A) and (T530S) point mutants failed to activate the reporter gene (data not shown). This result suggested that Ser526 and Thr530 are required for MEKK3-dependent regulation of the NF-κB promoter. Moreover, this result strongly suggested that Ser526 and Thr530 might be phosphorylation sites within the activation loop of MEKK3.

Ser526 or Thr530 were mutated to aspartic or glutamic acid, since such mutations have been shown to produce constitutively active kinases (21, 22). Experiments were performed in which S526A, S526D, S526E, T530A, T530D, and T530E MEKK3 were transfected into HEK293 EBNA cells, along with the NF-κB luciferase reporter gene. The S526A and T530A MEKK3 mutants were unable to activate transcription of the NF-κB luciferase reporter gene (Fig. 1B). However, the aspartic and...
glutamic acid mutants of Ser526 restored ~50% of wild-type MEKK3 activity, suggesting that the negative charge of the carboxyl group might mimic the negative charge of a phosphate moiety at this position. In contrast, the aspartic and glutamic acid mutants of Thr530 were unable to restore NF-κB luciferase activity, indicating that a negative charge at position 530 was not tolerated within the activation loop. In addition, mutation of the active site lysine to methionine (K391M) abrogated luciferase activity, indicating that the catalytic activity of MEKK3 was required for NF-κB-dependent luciferase activity. Thus, S526A and T530A MEKK3 were unable to activate transcription through the NF-κB promoter, although the active site lysine was retained, demonstrating that serine 526 and threonine 530 are important for MEKK3-dependent transcription.

**Activation Loop Mutants S526A and T530A MEKK3 Is Unable to Activate the MEK and ERK Pathway**—Overexpression of MEKK3 results in the activation of several MAP kinases that include the ERK, JNK, p38, and ERK5 pathways. Due to the intriguing biological activity of the S526A and T530A activation loop point mutants in the NF-κB luciferase assay, we chose to investigate the role of these point mutants in activating the ERK, JNK, and p38 pathways. First, we investigated the ERK MAPK pathway. HEK293 EBNA cells were transiently transfected with MEKK3, catalytically inactive MEKK3, and the seven activation loop point mutants to determine whether the activation loop point mutants were capable of activating the ERK pathway. After 48 h, cellular extracts were prepared and resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for phosphorylated ERK. Consistent with the luciferase assays described above, expression of (S526A) and (T530A), as well as catalytically inactive MEKK3, did not result in phosphorylation of ERK1 and -2 (Fig. 2, top). In contrast, expression of MEKK3, as well as the S511A, T516A, T520A, T522A, and T528A point mutants resulted in ERK activation. In addition, the constitutively active mutants of Ser526, S526D and S526E, also yielded a robust phosphorylation of the ERKs, whereas the constitutively active mutants of Thr530, T530D and T530E, were not able to activate the ERKs (data not shown), which was consistent with the luciferase assay previously shown. The nitrocellulose membrane was subsequently reprobed for ERK1 and -2 as a loading control, thereby demonstrating that differences in ERK phosphorylation were due to MEKK3 activity and not ERK expression. Previous work has shown that ERK1 and -2 are phosphorylated and activated by MEK1 and -2 (23). To determine whether expression of MEKK3 affected MEK phosphorylation, the experiment described above was also probed for phosphorylated MEK1 and -2. Consistent with the ERK data, cells transfected with the S526A and T530A point

**FIGURE 1. Regulation of luciferase activity by MEKK3.** A, schematic representation of the mouse MEKK3 activation loop showing the glycine-rich region of subdomain I, which begins at glycine 369 and constitutes a part of the nucleotide-binding domain of the catalytic domain of MEKK3. The active site lysine (K391) in subdomain II is critical for catalysis and hydrolysis of the γ-phosphate of ATP. The boundaries of the activation loop are defined by the DFG motif of subdomain VII and the SPE motif of subdomain VIII. The mouse and human isoforms of MEKK3 express serine and threonine at identical positions within the activation loop, which have been mutated to alanine for this study and are numbered according to the mouse sequence. The amino-terminal domain consisting of amino acids 1–350 is referred to as the regulatory domain, although no specific function has been ascribed to this domain at this time. B, constitutively active mutants of Ser526 regulate NF-κB luciferase activity. HEK293 EBNA cells were transfected with cDNAs that encode FLAG-tagged MEKK3 or the indicated point mutants with a cDNA that encodes the firefly luciferase gene downstream of the NF-κB promoter element. Cell extracts were prepared 48 h after transfection, and 10 μg was used to measure luciferase activity. C, in addition, 50 μg of cell extract was resolved by SDS-PAGE and immunoblotted (IB) using the monoclonal antibody that recognizes the FLAG epitope. The results are from a representative experiment.
Serine 526 Is Phosphorylated in the Activation Loop of MEKK3

FIGURE 2. Regulation of the MEK/ERK pathway by MEKK3. HEK293 EBNA cells were transfected with CDnas that encode FLAG-tagged MEKK3 or the indicated point mutants. After 48 h, cell extracts were prepared, and 50 µg was resolved by SDS-PAGE and immunoblotted (8) using antibodies that recognize phospho-ERK1/2, ERK1/2, phospho-MEK1/2, MEK1/2, and MEKK3.

Activating JNK—MEKK3 as indicated in Fig. 3B. After 48 h, 40 µg of cell extract was incubated with GST-c-Jun to precipitate endogenous JNK. The precipitated proteins were resolved by SDS-PAGE. Phosphorylated GST-c-Jun was identified by autoradiography (8) with monoclonal antibody that recognizes the FLAG epitope (A and B, bottom).

Mutants did not result in phosphorylation of MEK1 and -2 (Fig. 2, middle). The membrane was subsequently reprogrammed for MEK1 and -2 as a loading control. In addition, the membrane was also probed for MEKK3 expression by using the monoclonal antibody that recognizes the FLAG epitope, which demonstrated similar transfection efficiencies for each of the mutants. It is interesting to note that K391M, S526A, and T530A MEKK3, which are the three mutants that were unable to activate ERK and MEK phosphorylation, appeared as discrete polypeptides on the MEKK3 immunoblot, which typically indicates a lack of phosphorylation (Fig. 2, bottom, lanes c, e, and i). The reduced width of the immunoblotted protein bands probably reflects decreased phosphorylation of MEKK3 and not decreased expression (also see Fig. 5, lane g).

Activation Loop Mutants S526A and T530A MEKK3 Are Unable to Activate the p38 MAP Kinase—Since MEKK3 has been shown to activate the p38 MAP kinase (5) and function in an osmosensing scaffold with Rac, MKK3, and OSM (osmosensing scaffold for MEKK3) (6), we wanted to determine whether Ser526 and Thr530 were also involved in regulating the p38 MAP kinase. HEK293 EBNA cells were transfected with MEKK3, (S526A), and (T530A) MEKK3, and after 48 h, p38 MAP kinase was immunoprecipitated from cell extracts. An in vitro kinase assay was performed using GST-ATF2 as a substrate and [γ-32P]ATP. Proteins were resolved by SDS-PAGE, and phosphorylated GST-ATF2 was identified by autoradiography. Consistent with previous studies, expression of MEKK3 resulted in activation of p38 MAP kinase (Fig. 3, lane b), whereas transfection of an empty vector resulted in no p38 activity (lane a). A similar phosphorylation pattern was observed from nontransfected HEK293 EBNA cells treated with 0.4 M sorbitol (lane f). However, expression of S526A and T530A MEKK3 did not activate p38 MAP kinase (lanes c and d), although the expression of MEKK3 (Fig. 3, bottom, lane b) or the protein mutants were similar (lanes c and d). These results demonstrate that Ser526 and Thr530 are critical for MEKK3-dependent p38 MAP kinase activation.

Activation Loop Mutants S526A and T530A MEKK3 Are Unable to Activate JNK—MEKK3 has been shown to activate JNK (3, 5), and therefore we wanted to determine whether Ser526 and Thr530 were required to activate JNK. HEK293 EBNA cells were transfected with an empty vector as a control or constructs encoding various forms of MEKK3, as indicated in Fig. 3B. After 48 h, 40 µg of cell extract was incubated with GST-c-Jun to precipitate endogenous JNK. After washing the precipitated proteins with buffer, the GST-c-Jun/JNK protein complex was incubated with [γ-32P]ATP for 20 min, and then the proteins were resolved by SDS-PAGE. Phosphorylated GST-c-Jun was visualized by autoradiography. Expression of MEKK3 resulted in a robust activation of JNK (Fig. 3, lane b), whereas expression of an empty vector as a control (lane a), catalytically inactive MEKK3 (lane c), or the activation loop point mutants (lanes d and e) resulted in very little JNK activation. In summary, we demonstrate that the catalytic activity of MEKK3 is required to activate the ERK, p38, and JNK MAP kinases. In addition, mutation of Ser526 and Thr530 to alanine, while retaining the active site lysine at position 391, also produced catalytically inactive point mutants of MEKK3. Thus, Ser526 and Thr530 are critical amino acids within the activation loop of MEKK3.

Autophosphorylation of MEKK3 at Ser526—The Ser526 and Thr530 point mutants of MEKK3 clearly demonstrated that these two amino acids are critical for activity. However, it is unclear whether these amino acids are required to maintain the structural integrity of the activation loop or whether Ser526 and Thr530 are phosphorylation sites. In an attempt to address this question, we generated phosphospecific antibodies using peptide sequences that correspond to phospho-Ser526 and phospho-Thr530 of MEKK3. To evaluate the specificity of the phosphospecific antibodies, HEK293 EBNA cells were transfected with MEKK3, catalytically inactive MEKK3, S526A MEKK3, and T530A MEKK3, and not decreased expression. However, expression of S526A and T530A MEKK3 did not activate p38 MAP kinase (lanes c and d), although the expression of MEKK3 (Fig. 3, bottom, lane b) or the protein mutants were similar (lanes c and d). These results demonstrate that Ser526 and Thr530 are critical for MEKK3-dependent p38 MAP kinase activation.

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FIGURE 3. Regulation of JNK and p38 MAP activity by MEKK3. HEK293 EBNA cells were transfected with CDnas that encode FLAG-tagged MEKK3 or the indicated point mutants. To measure p38 MAP kinase activity, p38 was immunoprecipitated (IP) from cell extracts and then incubated with recombinant GST-ATF-2 and [γ-32P]ATP. The reactions were terminated by the addition of Laemmli sample buffer, and the proteins were resolved by SDS-PAGE. Phosphorylated GST-ATF-2 was identified by autoradiography (A, top). As a control, nontransfected HEK cells were treated with 0.4 M sorbitol for 30 min prior to immunoprecipitation of p38 MAP kinase. To measure JNK activity, cell extracts were incubated with GST-c-Jun to precipitate endogenous JNK. To the precipitated proteins was added [γ-32P]ATP, the kinase reactions were terminated with Laemmli sample buffer, and the proteins were resolved by SDS-PAGE. Phosphorylated GST-c-Jun was identified by autoradiography (B, top). An aliquot of the cell extract (50 µg) that corresponds to the lanes in the top was resolved on a separate gel and immunoblotted (8) with monoclonal antibody that recognizes the FLAG epitope (A and B, bottom).
MEKK3. After 48 h, cell extracts were prepared, and 50 μg of each extract was resolved by SDS-PAGE and immunoblotted with the phosphospecific antibodies. The anti-phospho-Ser526 antibody recognized MEKK3 (Fig. 4A, lane b), whereas cell extracts transfected with an empty vector showed no immunoreactivity, except a nonspecific protein of ~90 kDa (lanes a–e). Importantly, the phospho-Ser526 antibody showed no immunoreactivity toward cell extracts expressing SS26A MEKK3 (lane d), indicating that the antibody was specific for phospho-Ser526. In addition, catalytically inactive MEKK3 was not detected by the phospho-Ser526 antibody (lane c), strongly suggesting that phosphorylation of Ser526 occurs via autophosphorylation of MEKK3 and not by an upstream kinase. If phosphorylation of MEKK3 occurred via an upstream kinase that is endogenously expressed in HEK293 cells, one would expect phosphorylation of catalytically inactive MEKK3 at Ser526. It should be emphasized that the catalytically inactive mutant of MEKK3 can be phosphorylated at Ser166, which demonstrates that this mutant form of MEKK3 retains its structure for phosphorylation by another kinase (12), and presumably the structurally features surrounding Ser526 in the catalytically inactive mutant resemble wild-type MEKK3. This result demonstrates that the catalytically inactive mutant of MEKK3 retains the structural features needed for phosphorylation by another kinase and indicates that phosphorylation of Ser526 occurs by autophosphorylation. Finally, it is interesting to note that T530A MEKK3 was recognized by the anti-phospho-Ser526 antibody (lane e), albeit to a lesser extent than wild type MEKK3. This result suggests that Thr530 is not essential for phosphorylation of Ser526, although Thr530 is required for signaling to downstream substrates. Reprobing the immunoblot with a monoclonal antibody that recognizes the FLAG epitope of transfected MEKK3 indicated that similar levels of the MEKK3 proteins were expressed in the cells (Fig. 4A, bottom).

Similar experiments were performed using affinity-purified antisera generated against a peptide containing phospho-Thr530 of MEKK3 (data not shown). Antisera from three different rabbits were affinity-purified and tested for immunoreactivity against phospho-Thr530. Although the sera tested positive against the antigen that was used to immunize the rabbits, all of the antisera tested negative for phosphorylation at Thr530 when MEKK3 was overexpressed in HEK293 EBNA cells. Thus, based on these data and the fact that the (T530D) and (T530E) mutants were unable to activate MEKK3-dependent signaling pathways, we conclude that Thr530 is not phosphorylated in the activation loop of MEKK3.

Phosphorylation of Endogenous MEKK3 at Ser526—Having developed an antibody that recognized phospho-Ser526, we could determine whether Ser526 is phosphorylated in vivo. Since MEKK3 functions in the p38 MAPK pathway (6), osmotic stress was used to activate endogenous MEKK3. NIH3T3 cells were deprived of serum for 18 h and then treated with 0.4 M sorbitol for 30 min (lanes c and d). Cell extracts (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibody as described in A.

Dephosphorylation of Ser526—By developing an antibody that recognized phospho-Ser526, we could determine whether Ser526 was sensitive to dephosphorylation. To test this hypothesis, HEK293 EBNA cells were transfected with FLAG-tagged MEKK3 or an empty vector, and cell extracts were prepared in lysis buffer lacking phosphatase inhibitors. Cell extracts were incubated at 30 °C for the indicated times and, where indicated, with 10 μg/ml okadaic acid (OA), or both. Proteins were resolved by SDS-PAGE and immunoblotted (IB) with anti-phospho-Ser526 rabbit polyclonal antibody (top). The same nitrocellulose membrane was stripped and probed with antibody that recognizes the amino terminus of MEKK3 (bottom).
Serine 526 Is Phosphorylated in the Activation Loop of MEKK3

was supplemented with 1.5 μM of 12 mM Tris, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT, and 0.12 mg/ml bovine serum albumin. Then the cell extracts were incubated at 30 °C for 1 h. Where indicated, phosphatase reactions also included 10 mM NaF or 100 nM okadaic acid. As we have previously shown, expression of MEKK3 in HEK293 EBNA cells resulted in phosphorylation of MEKK3 at Ser526 (Fig. 5, top, lane f). In this experiment, we demonstrate that incubation of MEKK3 in the HEK293 EBNA cell extract resulted in complete dephosphorylation of MEKK3 (Fig. 5, top, lane g). Immunoblotting the same nitrocellulose membrane with antibody that recognizes the amino terminus of MEKK3 demonstrated that the lack of immunoreactivity in lane g was not due to degradation of MEKK3. In fact, the loss of phosphate from MEKK3 is reflected by the increased mobility and decreased molecular mass of MEKK3 by SDS-PAGE (Fig. 5, bottom, compare lane g with lane f). Moreover, serine/threonine phosphatase inhibitors such as NaF and okadaic acid were included in the phosphatase reactions and were highly effective at inhibiting the phosphatase activity in the HEK293 EBNA cell extracts (Fig. 5, top, lanes h–j). In summary, these data demonstrate that Ser526 of MEKK3, when incubated in cell extracts, is susceptible to dephosphorylation and that the phosphatase(s) responsible for this activity is sensitive to NaF and okadaic acid.

Dephosphorylation of Ser526 by PP2A Isoforms—Since we had established dephosphorylation of Ser526 when MEKK3 was incubated in cell extracts, we tested whether the protein serine/threonine phosphatase, PP2A, could dephosphorylate Ser526. To obtain two different PP2A holoenzymes, HEK293 cells were transfected with the FLAG-tagged α or β isoform of the regulatory B subunit of PP2A. The A subunit is a scaffolding protein, whereas the C subunit is the catalytic protein of PP2A. The PP2A holoenzymes, consisting of either ABαC or ABβC subunits, were isolated by immunoprecipitation using the monoclonal antibody that recognizes the FLAG epitope. The PP2A holoenzymes were eluted from the Sepharose beads using the FLAG peptide at a concentration of 0.1 mg/ml as previously described (24). In a separate transfection, HEK293 EBNA cells were transfected with FLAG-tagged MEKK3, and after 48 h, cell extracts were prepared, and tagged MEKK3 was immunoprecipitated using the FLAG monoclonal antibody. Immunoprecipitated FLAG MEKK3 was incubated with 9 ng of PP2A holoenzymes, ABαC (Fig. 6, lanes b and c) or ABβC (Fig. 6, lanes d and e) for 30 or 60 min at 30 °C. Within 30 min, phospho-Ser526 was completely dephosphorylated by either PP2A holoenzyme. These results suggest that the protein phosphatase that dephosphorylates Ser526 from HEK293 EBNA cell extracts is probably an isoform of PP2A.

Dephosphorylation of Ser526 Affects MEKK3 Catalytic Activity toward MKK6—Since MEKK3 is a MAP3K that functions upstream of the p38 MAP kinase and we had shown the phospho-Ser526 was required for MEKK3-dependent activation of p38 (Fig. 3A), we tested whether phosphorylation of Ser526 was required for MEKK3 to directly phosphorylate a substrate like MKK6. The cDNA that encodes FLAG-MEKK3 was transfected into HEK293 EBNA cells, and after 48 h, Sepharose beads conjugated to the monoclonal antibody that recognizes the FLAG epitope were used for immunoprecipitation. Precipitated proteins were washed twice with 1 ml of PAN Nonidet P-40 buffer, once with 1 ml of PAN buffer, and aliquoted into two tubes. After centrifugation to collect the protein-bound beads, 100 μg of nontransfected HEK293 EBNA cell extract that was prepared with 10 mM NaF and 100 mM okadaic acid (Fig. 7, A (lanes a and b), B (lane b), and C (lane a)) or without phosphatase inhibitors (Fig. 7, A (lanes c and d), B (lane b), and C (lane b)) was added to each tube and incubated for 1 h at 30 °C. After the dephosphorylation reaction, 20% of the sample was removed to assess dephosphorylation of FLAG-MEKK3 by immunoblotting with antibody that recognizes phospho-Ser526 by immunoblotting with antibody that recognizes phospho-Ser526. The PP2A holoenzymes, consisting of ABαC or ABβC, were eluted from the Sepharose beads with the FLAG peptide, and then 2 ng were incubated with immunoprecipitated MEKK3 for the indicated times at 30 °C (lanes b–e). The reactions were terminated with Laemmli sample buffer, the proteins were resolved by SDS-PAGE and immunoblotted (IB) with anti-phospho-Ser526 antibody (top), and then the nitrocellulose membrane was reprobed with antibody that recognizes the amino terminus of MEKK3 (bottom).

FIGURE 6. PP2A isoforms dephosphorylate Ser526. HEK293 EBNA cell extracts were transfected with FLAG-tagged MEKK3 and immunoprecipitated with antibody that recognizes the FLAG epitope (lanes a–e). In a separate transfection, HEK293 cells were transfected with the FLAG-tagged Bα or Bβ subunit of PP2A. The PP2A holoenzymes consisting of ABαC or ABβC were eluted from the Sepharose beads with the FLAG peptide, and then 2 ng were incubated with immunoprecipitated MEKK3 for the indicated times at 30 °C (lanes b–e). The reactions were terminated with Laemmli sample buffer, the proteins were resolved by SDS-PAGE and immunoblotted (IB) with anti-phospho-Ser526 antibody (top), and then the nitrocellulose membrane was reprobed with antibody that recognizes the amino terminus of MEKK3 (bottom).

FIGURE 7. Dephosphorylation of Ser526 decreases MEKK3 catalytic activity toward MKK6. HEK293 EBNA cells were transfected with FLAG-tagged MEKK3, and immunoprecipitation was performed using Sepharose beads conjugated to the monoclonal antibody that recognizes the FLAG epitope (A). After the immunoprecipitation, the protein-bound beads were washed with buffer and aliquoted into two separate Eppendorf tubes, and the beads were collected by centrifugation. One tube was incubated with 100 μg of HEK293 EBNA cell extract prepared with (A, lanes a and b), B (lane a), and C (lane a) or without phosphatase inhibitors for 1 h at 30 °C (lanes c and d), B (lane b), and C (lane b). To assess the dephosphorylation of FLAG-MEKK3, 20% of each sample was collected, and Laemmli sample buffer was added to terminate the dephosphorylation reaction. These samples were resolved by SDS-PAGE and first immunoblotted (IB) with antibody that recognizes phospho-Ser526 (B), and then the membrane was reprobed with antibody that recognizes the amino terminus of MEKK3 (C). To the remaining samples, cell extract was removed after centrifugation of the beads, and the FLAG MEKK3-bound beads were washed twice with PAN buffer and aliquoted into two separate tubes again (A, lanes a and b and lanes c and d). An in vitro kinase assay was performed with (lanes b and d) or without (lanes a and c) 10 μg of KA-MKK6 as substrate and γ-32P]ATP. The kinase assays were terminated by the addition of Laemmli sample buffer, and the proteins were resolved by SDS-PAGE and stained with Coomassie Blue (data not shown), followed by autoradiography (A). This is a representative figure of four separate experiments.
Serine 526 Is Phosphorylated in the Activation Loop of MEKK3

Serine 526 is required for association with 14-3-3. HEK293 EBNA cells were transfected with FLAG-tagged MEKK3 or various point mutants (lanes b–e), cell extracts were prepared, and an antibody that recognizes the FLAG epitope was used to immunoprecipitate the MEKK3 proteins. The samples were resolved by SDS-PAGE and immunoblotted (top) with antibody that recognizes phospho-Ser526 (top), and then the membrane was reprobed with antibody that recognizes the amino terminus of MEKK3 (middle). The bottom portion of the membrane was probed with 14-3-3 antibody that recognizes amino acids 3–21 of the human isoform of 14-3-3β. This sequence is highly conserved within the 14-3-3 family of proteins. Since we were able to demonstrate dephosphorylation of Ser526 as shown in Fig. 5, an experiment was designed to determine whether the association between MEKK3 and 14-3-3 would prevent dephosphorylation of Ser526. HEK293 EBNA cells were transfected with FLAG-MEKK3, and cell extracts were prepared in the absence of OKadaic acid and sodium fluoride. Then the cell extracts were incubated at 4°C with GST-14-3-3ε or GST-lamin that was expressed and purified from bacteria, as previously described. After 1 h, the GST fusion proteins and associated proteins were collected by centrifugation and washed twice with 1 ml of PAN-Nonidet P-40 and PAN buffers to remove nonspecific proteins. Precipitated proteins were resolved by SDS-PAGE and immunoblotted using the anti-phospho-Ser526 antibody. 14-3-3ε precipitated FLAG-MEKK3 while phosphorylated at Ser526 (Fig. 9, top, lane a), whereas the lamin fusion protein was unable to precipitate FLAG-MEKK3 (lane g), as we have previously reported (28).

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Serine 526 Is Phosphorylated in the Activation Loop of MEKK3

amount of FLAG-MEKK3 that was used in each experimental condition was similar, as determined by immunoblotting with antibody that recognizes the amino terminus of MEKK3 (Fig. 9, bottom, compare lanes a–f with lanes i–k). In summary, the association between FLAG-MEKK3 and 14-3-3ε inhibits dephosphorylation of Ser526. Thus, given the importance of phospho-Ser526 in regulating MEKK3 activity, it appears that the interaction between MEKK3 and 14-3-3ε is critical for maintaining a catalytically active form of MEKK3.

DISCUSSION

MEKK3 is a serine/threonine kinase of the MAP3K family that has been implicated as an upstream regulator in the signaling pathways for a number of MAP kinases (3–7) as well as the tumor necrosis factor signaling pathway (9, 20, 29–31). Most studies have focused on the signaling properties of MEKK3 after stable or transient introduction of this kinase into target cells or by suppression of MEKK3 expression by small interfering RNA. However, little work has been done to directly measure MEKK3 activity. To address this issue, we set out to identify phosphorylation sites in the activation loop of MEKK3, so that phosphospecific antibodies could be used to monitor MEKK3 activity.

In this study, we demonstrate that Ser526 is the only phosphorylation site within the activation loop of MEKK3 and that phosphorylation occurs in response to exogenous stimuli like osmotic stress. In addition, whereas mutation of other putative phosphorylation sites did not significantly alter MEKK3 activity, mutation of Thr530 to a conserved alanine or to the phosphomimetic amino acids, aspartate or glutamate, was not tolerated, suggesting that Thr530 might play an important role in maintaining the structural integrity of the activation loop. Although the crystal structure of the catalytic domain of MEKK3 has not been resolved, the crystal structure of the catalytic subunit of PKA provides insights as to the orientation of the activation loop of MEKK3. Two critical amino acids between subdomains VII and VIII of the catalytic subunit of PKA provides insights as to the orientation of the activation loop of MEKK3. The crystal structure of PKA that is bound to subdomain VII and VIII of the catalytic subunit of PKA, Thr197 and Thr201, are localized at positions equivalent to those of Ser526 and Thr530 of MEKK3, respectively. In PKA, Thr197 is phosphorylated and critical for activity, whereas Thr201 is also critical for activity but not a phosphorylation site (32). In this study, we have demonstrated that Ser526 of MEKK3 is phosphorylated and critical for catalytic activity, like Thr197 of PKA. We have also shown that Thr530, which corresponds to Thr201 of PKA, is required for MEKK3 activity and not likely to be a phosphorylation site. This result suggests that Thr530 is critical to maintain the structural integrity of the activation loop of MEKK3. In the crystal structure of PKA that is bound to substrate peptide, Thr201 of the PKA catalytic subunit interacts with Asp166 but only in a substrate-dependent manner (32). Given the similarities between the PKA and MEKK3 activation loops, it is quite possible that Thr530 of MEKK3 interacts with Asp489, which corresponds to Asp166 of PKA. The fact that alanine or negatively charged amino acids are not tolerated at position 530 supports the notion that the hydroxyl moiety of Thr530 interacts with a negatively charged amino acid, like Asp489. The crystal structure of the MEKK3 catalytic domain while bound to substrate peptide should confirm this prediction.

The development of an antibody that recognizes phospho-Ser526 provides compelling evidence that Ser526 is a phosphorylation site within the activation loop of MEKK3. Further evidence that a negative charge at Ser526 is critical for MEKK3 activity comes from experiments using the S526D and S526E mutants of MEKK3, which activated both the ERK and NF-kB luciferase activity. In contrast, negatively charged amino acids at Thr530 produced inactive mutants of MEKK3. Moreover, attempts to detect phospho-Thr530 of MEKK3 with a phosphospecific antibody were unsuccessful (data not shown), which supports the conclusion that Thr530 is not a phosphorylation site. However, although Thr530 is not a phosphorylation site, Thr530 is required for the phosphotransfer reaction catalyzed by MEKK3.

The catalytically inactive mutant of MEKK3, which has the active site lysine mutated to methionine (K391M), was not phosphorylated on Ser526. Based on this observation, our results suggest that phosphorylation of Ser526 occurs by an autophosphorylation mechanism. If phosphorylation of Ser526 had occurred in the K391M MEKK3 mutant, such a result would have indicated that an upstream kinase in the signaling cascade, such as a mitogen-activated protein kinase kinase kinase, kinase, phosphorylates Ser526. But this result was never observed in these studies. It is interesting to note that although Thr530 is required for MEKK3-dependent MAP kinase activity, autophosphorylation of Ser526 does not require Thr530, albeit efficient autophosphorylation of Ser526 may require Thr530. Nonetheless, our results suggest that the autophosphorylation of MEKK3 may occur via a different mechanism from phosphorylation of downstream kinases, such as the MAP2K family. Since the MKKs require dual phosphorylation for efficient catalytic activity, it is possible that Thr530 is necessary to orient the activation loop of MEKK3 for dual phosphorylation of MKks, whereas Thr530 is not necessary for phosphorylation of Ser526. Additional mechanistic studies will be needed to delineate the difference between those phosphorylation events.

Dephosphorylation of Ser526 was readily accomplished by using purified PP2A holoenzymes. Moreover, the presence of PP2A inhibitors such as okadaic acid or sodium fluoride in cell extracts containing transfected MEKK3 prevented dephosphorylation of Ser526, and the absence of phosphatase inhibitors resulted in dephosphorylation of Ser526. The reversible nature of Ser526 phosphorylation further supports the fact that a post-translational modification of Ser526 is physiologically relevant for MEKK3 activity, especially under conditions of osmotic stress. The study described herein also demonstrates that phospho-Ser526 is also required for efficient phosphorylation of downstream substrates, such as MKK6. Importantly, dephosphorylation of Ser526 is prevented by the interaction with 14-3-3 protein, which is consistent with the previously defined role of 14-3-3 proteins binding to phosphorylated serine or threonine (33). However, phospho-Ser526 is not localized within a consensus 14-3-3 binding site as described by Yaffe et al. (34).

Although we know that 14-3-3 binding to MEKK3 prevents dephosphorylation of MEKK3, we do not know whether phospho-Ser526 contributes directly to the binding with 14-3-3 protein. Our previous study using the amino terminus of MEKK3 as bait in the yeast two-hybrid system suggests that Ser526 is not directly involved in 14-3-3 binding, since we have shown that the amino terminus is sufficient to interact with 14-3-3 proteins (28). Thus, perhaps other phosphorylation sites on MEKK3, such as Ser466 and/or Ser467, are needed for binding with 14-3-3 (12) and the tertiary structure of MEKK3, when bound to 14-3-3, does not allow phosphatases to access phosphorylated Ser526.

Future studies must now be directed toward understanding the mechanism by which MEKK3 autophosphorylation occurs and, conversely, how protein phosphatases like PP2A dephosphorylate Ser526. It is clear that the interaction between MEKK3 and 14-3-3 proteins helps maintain phosphorylation of Ser526, but what causes the dissociation of 14-3-3 proteins from phosphorylated MEKK3 is a question whose answer will probably allow us to better understand how MEKK3 functions in the cell. In summary, Ser526 is an activation loop phosphorylation site that is critical for MEKK3 activity, much as phosphorylation of the activation loop of Thr197 of the catalytic subunit of PKA functions as a molecular switch for enzymatic activity (35). Moreover, Ser526 pro-
provides a molecular target to readily assess MEKK3 activity under different physiological conditions.

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