Phosphorylation of MEKK3 at Threonine 294 Promotes 14-3-3 Association to Inhibit Nuclear Factor κB Activation

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The protein kinase MEKK3 is essential for tumor necrosis factor α (TNFα)- and lipopolysaccharide-induced activation of nuclear factor κB, although the mechanism by which TNF receptor 1 and Toll-like receptors regulate MEKK3 is largely unknown. In this study we have identified MEKK3 Thr294 as a novel site of phosphorylation that regulates MEKK3 binding with 14-3-3. Phosphorylation of MEKK3 at Thr294 was observed for both endogenous and ectopically expressed MEKK3. Mutation of Thr294 to alanine abolished 14-3-3-MEKK3 association and incubation with phosphorylated peptides mimicking Thr(P)294 competed for 14-3-3 binding. Mutation of Thr294 did not alter Ser226 phosphorylation within the activation loop. However, expression of T294A MEKK3 elevated TNFα-stimulated NF-κB transcriptional activity, suggesting that Thr294 phosphorylation and 14-3-3 binding negatively regulate MEKK3. Stimulation with TNFα or lipopolysaccharide caused a rapid decrease in Thr294 phosphorylation of endogenous MEKK3 and subsequent loss of 14-3-3 association. Thus, this study identifies a potentially important regulatory step in MEKK3 signaling via dephosphorylation of Thr294, which reduces 14-3-3 binding correlating with MEKK3 pathway activation.

Protein kinases are key regulators of extracellular cues that illicit gene expression. One group of protein kinases involved in a wide array of cellular functions is the mitogen-activated protein kinase (MAPK)3 family consisting of the well characterized extracellular regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (also called stress-activated protein kinase), p38 MAPK, and the Big MAPK/ERK5. These family members are all activated by linear phosphorylation cascades involving upstream protein kinases called MAPK kinase and MAP3K. Numerous MAP3Ks have been identified, including MEKK1, MEKK2, MEKK3, MEKK4, tumor progression locus 2, and transforming growth factor-B-activated kinase 1.

MAP3Ks are emerging as important regulators of NF-κB. NF-κB belongs to a family of transcription factors that are essential mediators of pro-inflammatory signals involved in innate immunity. The mechanism of NF-κB regulation has been the subject of intense research for the past decade and is becoming better understood. NF-κB is sequestered to the cytoplasm by the inhibitor protein of κB (IκB). NF-κB can translocate to the nucleus following phosphorylation and degradation of IκB by IκB kinase (IKK). Activation of IKK occurs rapidly following pro-inflammatory receptor activation, and both TNFα- and LPS-induced NF-κB activation is lost in mouse embryonic fibroblasts devoid of MEKK3 (1). IκK might be directly phosphorylated by MEKK3 (2), but the mechanism of how TNF receptor 1 and TLR4 regulate MEKK3 phosphorylation of IKK in response to TNFα or LPS is not understood.

A potential mechanism of MEKK3 regulation is through interaction with 14-3-3 molecules. 14-3-3 proteins are recognized as important cellular regulators by facilitating the interaction between phosphorylated proteins (3–5) and are involved in regulating a diverse set of cellular proteins by altering activity, location, and stabilization (6, 7). The interaction between another MAP3K, Raf-1, and 14-3-3 is an example of this complex regulation, with both activating and inactivating functions. For example, Raf-1 is inactivated by phosphorylation at Ser259, which promotes the association with 14-3-3 proteins and stabilizes Raf-1 in an inactive conformation that cannot be recruited and activated by Ras (8–10). In contrast, Ser621 phosphorylation mediates distinct 14-3-3 binding, which stabilizes Raf-1 in an active conformation (11–13). MEKK3 has been shown to associate with 14-3-3 (14, 15), although the site of interaction is not known, and therefore it is not clear what function 14-3-3 association might play. Here we show that 14-3-3 binding occurs at phosphorylated Thr294 of MEKK3. Agonists of the MEKK3 pathway, including TNFα and LPS, caused a reduction in Thr294 phosphorylation for endogenous MEKK3 leading to a loss of 14-3-3 binding. Furthermore, mutation of Thr294 to alanine led to elevated TNFα-stimulated NF-κB activation. Therefore, this study suggests that pro-inflammatory molecules such as TNFα and LPS contribute to MEKK3-dependent NF-κB activation by modulating Thr294 phosphorylation and 14-3-3 binding.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK 293, HeLa, and RAW 264.7 cells were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C, 5% CO2, and humidity.
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Plasmids and Mutagenesis—Human MEKK3 cDNA was cloned from IMAGE clone 7939519 by PCR and inserted into pCMV10-3xFLAG to introduce an N-terminal FLAG epitope. Mutagenesis of pCMV10-3xFLAG-MEK3 was performed using QuikChange kit (Stratagene), and the mutations were sequence-verified.

**cDNA Transfection**—HEK 293 or HeLa cells were plated onto 35-mm-diameter dishes at 80% confluency and transfected with 100–500 ng of cDNA using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Transfection medium was removed and replaced with complete Dulbecco’s modified Eagle’s medium overnight.

Cell Lysis, Immunoprecipitation, and Immunoblotting—The cells were lysed in 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 25 mM NaF, 25 mM β-glycerophosphate, 5 mM EDTA, 0.05% SDS, 100 mM okadaic acid and protease inhibitors. Anti-FLAG M2 antibody conjugated to agarose beads (Sigma) were added to lysates and incubated overnight at 4 °C. The beads were washed three times with lysis buffer, and proteins were eluted with 200 μl of lithium dodecyl sulfate sample buffer heated to 70 °C for 10 min. Portions of the lysates prior to immunoprecipitation were also boiled with lithium dodecyl sulfate-containing sample buffer. Lysates and immunoprecipitations were fractionated on 8% SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane, blocked in 5% skim milk for 30 min, and probed with the appropriate antibody overnight at 4 °C. Secondary decoration with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies was performed at room temperature for 1.5 h. The proteins were visualized using ECL according to the manufacture’s protocol (Amer sham Biosciences). For blotting using the Licor Odyssey infrared imager, the membranes were probed with IR700 anti-rabbit and IR800 anti-mouse secondary antibodies. FLAG was stained with anti-FLAG M2 mouse monoclonal (Sigma), and 14-3-3 isoforms were detected with anti-14-3-3-β (H8; Santa Cruz Biotechnology).

Metabolic Labeling—HEK 293 cells were plated onto 35-mm-diameter dishes at 80% confluency and transfected with 500 ng of pCMV10-FLAG-MEKK3 using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. After 24 h, the cells were placed in phosphate-free Dulbecco’s modified Eagle’s medium with 1 mCi/ml 32P-labeled orthophosphate at 37 °C for 4 h. FLAG-MEKK3 was immunoprecipitated from detergent-solubilized lysates and fractionated on an 8% gel. 32P-Labeled MEKK3 was detected by autoradiography.

Tryptic Digestion, Two-dimensiona l Phosphopeptide Mapping, and Phosphoamino Acid Analysis—Metabolically 32P-labeled FLAG-MEKK3 was isolated as described above, excised from the gel, and digested with 10 μg/ml tosylphenylalanyl chloromethyl ketone-treated trypsin (Promega) in 50 mM (NH4)HCO3, pH 7.8, overnight at 37 °C. The gel fragments were pelleted by centrifugation, and the remaining supernatant was transferred to clean tubes and dried under vacuum. Dried peptides were resuspended in 50 μl of performic acid, incubated on ice for 2 h, and dried under vacuum. The peptides were washed with diminishing volumes of water and resuspended in 5 μl of pH 1.9 electrophoresis buffer. Electrophoresis was performed on 200-μm microcrystalline cellulose plates (Kodak) at 1000 V, 7 °C for 30 min. The plates were chromatographed in the second dimension in chromatography buffer (n-butanol/pyridine/acetic acid/water, 32.5:25:5:20). The plates were dried, and phosphopeptides were visualized using an FX-Imager (Bio-Rad) or film autoradiography. If cold synthetic phosphopeptides were also run, these were visualized with ninhydrin staining. Phosphoamino acid analysis was performed by hydrolyzing 32P-labeled MEKK3 in 500 μl of 6 N HCl heated to 110 °C for 60 min. The HCl was removed under vacuum, and the phosphoamino acids were washed with diminishing volumes of water. Separation was performed on cellulose plates using buffer consisting of 0.5% pyridine and 5% acetic acid at 1500 V at 7 °C for 20 min in one direction, and then the plate was allowed to dry. Once dry, phosphoamino acids were separated in a second dimension using pH 3.5 buffer at 1600 V at 7 °C for 13 min. 32P-Labeled phosphoamino acids were detected by autoradiography. In each of the samples, 1 μg of a mixture of phospho-γ- serine, phospho-δ-threonine, and phospho-δ-tyrosine was also added that was visualized by ninhydrin staining.

**Peptide Competition Assay**—The Thr294 peptide (DGGRTFPRIR) and phosphopeptide (DGGRtFtFPRIR) corresponding to the sequence surrounding Thr294 were synthesized by Genscript Corp. A scrambled phosphopeptide RFPFDpTGRRI was used as a negative control. Lysates containing FLAG-MEKK3 were incubated with 200 μM of peptide or phosphopeptide where indicated. Anti-FLAG M2 agarose was added, and the mixtures were incubated 4 °C. The beads were washed three times, and proteins were eluted and resolved by SDS-PAGE. Co-immunoprecipitated 14-3-3 was detected using a pan-specific anti-14-3-3-β antibody (Santa Cruz).

In Vitro Kinase Assay—MEKK3 was immunoprecipitated from HEK 293 cells and washed three times with solubilization buffer (200 mM Tris, pH 7.4, 0.5% Nonidet P-40, 0.25 mM NaCl, 250 mM β-glycerophosphate, 250 mM NaF, 2 mM Na3VO4, 100 mM okadaic acid, protease inhibitor mixture, and twice with kinase assay buffer (25 mM Tris, pH 7.4, 25 mM MgCl2, 1 mM EGTA, 2 mM dithiothreitol, 25 mM β-glycerophosphate, 1 μg/ml microcystin LR). The reactions were initiated by adding [γ-32P]ATP and incubating for 20 min at 30 °C. The reactions were stopped by adding an equal volume of 2× sample buffer and boiling. The proteins were resolved by SDS-PAGE, transferred to polyvinylidene difluoride, and immunoblotted with anti-FLAG M2 antibody or anti-Ser(P)526 antibody. Radiolabeled proteins were visualized by phosphorimaging.

**NF-κB Reporter Assay**—HeLa cells were transfected with 500 ng of FLAG-MEKK3, T294A-MEKK3, or empty vector along with 500 ng of ELAM-Luciferase and 200 ng of pCMV-β-galactosidase. After 24 h, the cells were stimulated with 10 ng TNFα for 4 h, and luciferase in the cell extracts was measured (Promega). All of the values were normalized to β-galactosidase activity.

**RESULTS**

MEKK3 Requires Autophosphorylation for Association with 14-3-3—To better understand how MEKK3 is regulated by association with 14-3-3, we monitored the co-immunoprecipitation of 14-3-3 with MEKK3 harboring various mutations. Consistent with others, we found that MEKK3 co-immunopre-
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Identification of Thr294 as a Novel Site of Phosphorylation—
To identify the site of 14-3-3 interaction, we first began by substituting predicted serine-containing sites with alanine. This approach ruled out Ser166, Ser236, Ser250, Ser337, and Ser357, because mutation of all these residues to alanine did not abolish MEKK3 co-immunoprecipitation (Fig. 1B). The profile of 14-3-3 co-immunoprecipitation suggested that MEKK3 could be phosphorylated on residues that experience autophosphorylation. To identify these additional unknown site(s) of phosphorylation, we expressed WT MEKK3 and K391M MEKK3 in cells labeled with [32P]orthophosphate (Fig. 2A). We then digested [32P]-labeled MEKK3 with trypsin and resolved the tryptic fragments by two-dimensional electrophoresis and cellulose chromatography (Fig. 2B). A major peptide, labeled A, was absent in the K391M MEKK3, suggesting that A was a peptide that undergoes autophosphorylation. To help identify peptide A, we performed phosphoamino acid analysis on [32P]-labeled WT MEKK3 (Fig. 2C). This revealed a mixture of phosphoserine and phosphothreonine, indicating that MEKK3 was phosphorylated in cells on both amino acids. We did not detect tyrosine phosphorylation. Serine phosphorylation was expected; however, threonine phosphorylation was surprising and novel.

FIGURE 1. Association of 14-3-3 requires MEKK3 catalytic activity and is independent of Ser166, Ser236, Ser250, Ser337, and Ser357. A, HEK 293 cells were transfected with WT MEKK3, K391M MEKK3, S526A MEKK, or empty vector. After 24 h, the cells were solubilized, and FLAG-MEKK3 was immunoprecipitated (IP) with anti-FLAG M2-conjugated agarose. The proteins were resolved by SDS-PAGE and immunoblotted with anti-FLAG antibody or anti-14-3-3 antibody (which recognizes the family of 14-3-3 proteins). B, HEK 293 cells were transfected with WT MEKK3, K391M MEKK3, TM (S166/S250/S337A compound mutation), S357A MEKK, or empty vector. After 24 h, the cells were solubilized, and FLAG-MEKK3 was immunoprecipitated with anti-FLAG M2-conjugated agarose. Proteins were resolved by SDS-PAGE and immunoblotted with anti-FLAG antibody or anti-14-3-3 antibody.

FIGURE 2. Identification of Thr294 as a site of phosphorylation. A, WT MEKK3 or K391 MEKK3 were expressed in HEK 293 cells for 24 h and then transferred for 4 h to phosphate-free medium containing 1 mCi/ml (32P)orthophosphate. FLAG-MEKK3 was immunoprecipitated with anti-FLAG M2-conjugated agarose, fractionated by SDS-PAGE, and visualized by autoradiography. B, 32P-labeled MEKK3 was hydrolyzed with HCl and resolved by two-dimensional electrophoresis. 32P-Labeled amino acids were visualized by autoradiography. Co-migrating cold amino acids were visualized by ninhydrin staining. C, 32P-labeled WT MEKK3 and K391M MEKK3 were digested with trypsin and resolved by two-dimensional electrophoresis/chromatography as described under “Experimental Procedures.” The synthetic phosphopeptide pTFPR (1 μg) was included in the WT MEKK3 sample and visualized by ninhydrin staining (indicated by the circle at spot A). D, preimmune serum or serum from rabbits immunized for 56 days with the synthetic phosphopeptide YNDGRRpTFPRIRR coupled to keyhole limpet hemocyanin were used to immunoblot lysates from cells expressing empty vector, WT MEKK3, K391M MEKK3, or T294A MEKK3. Total FLAG-MEKK3 was visualized using anti-FLAG antibody. E, HeLa cells were grown on coverslips and transfected with FLAG-MEKK3 or FLAG-T294A MEKK3. The cells were stained with anti-FLAG M2 antibody or purified anti-Thr(P)294 antibody and visualized by confocal microscopy.
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We examined the MEKK3 sequence to identify potential sites of threonine phosphorylation. The threonine residue at 294 within the sequence 291GRRTFPRI298 (of MEKK3 isoform 2; Thr345 of the longer MEKK3 isoform 1) was considered as a potential site of phosphorylation because of the arginine at -2, the phenylalanine at +1, and the proline at +2. This motif resembles the canonical phosphorylation motif for arginine/lysine-directed, basophilic kinases, and also resembles the Mode 1 site for 14-3-3 protein binding. To test whether this site was phosphorylated in cells, we included in the tryptic maps the synthetic peptide pTFPR, corresponding to the unique site was phosphorylated in cells, we included in the tryptic maps the synthetic peptide pTFPR, corresponding to the unique site for 14-3-3 protein binding. To test whether this site plays an important role in stabilizing 14-3-3-MEKK3 interaction. To verify that Thr294 was the site of 14-3-3 binding, we 14-3-3 co-immunoprecipitation (Fig. 3A, upper panel). Anti-14-3-3 antibody. This experiment was repeated three times with identical results. B, HEK 293 cells were transfected with WT MEKK3 for 24 h. Anti-FLAG M2 agarose was added to the cell lysates along with 200 μg of the peptide DGGRTFPRIR, phosphopeptide DGGRpTFPRIR, or scrambled phosphopeptide RPFpDTPGRR where indicated. After 2 h, the beads were washed, and bound 14-3-3 was visualized by immunoblotting with an anti-14-3-3 antibody.

these experiments established that Thr294 was a primary site of 14-3-3 protein interaction.

Generation of a Phosphospecific Ser526 Antibody—Mutation of Ser526 to alanine abolished 14-3-3 co-immunoprecipitation (Fig. 1A). Therefore, it remained possible that the dependence of Thr294 phosphorylation on 14-3-3 association could be a result of altered Ser526 phosphorylation. To monitor Ser526 phosphorylation, we generated a phosphospecific antibody to this site. Purified antibody from rabbits immunized with the synthetic peptide corresponding to Ser(P)526 detected WT MEKK3 but not SS26A MEKK3 (Fig. 4A). Furthermore, K391M MEKK3 was not detected by the anti-Ser(P)526 antibody. Consistent with previous reports that phosphorylation of this site occurs by autophosphorylation (16).

For MEKK3 containing the T294A mutation, Ser526 remained phosphorylated to a level similar to WT MEKK3; however, 14-3-3 co-immunoprecipitation was completely abolished (Fig. 4A). Also, conversion of Ser526 to a phosphomimetic aspartic acid (S526D) resulted in a partial rescue of Thr294 phosphorylation and 14-3-3 binding, consistent with previous reports that S526D partially rescues kinase activity (15, 16). Therefore, we conclude that Ser526 phosphorylation is constitutive and is likely required for autophosphorylation of
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Endogenous MEKK3 Is Phosphorylated at Thr<sup>294</sup> and Is Decreased by TNFα and LPS Stimulation—So far, these experiments have examined the phosphorylation of ectopically expressed MEKK3. We wanted to know whether endogenous MEKK3 was phosphorylated at Thr<sup>294</sup> and whether known stimuli of MEKK3 including TNFα and LPS regulate the phosphorylation of this site. Thus, we immunoprecipitated MEKK3 from RAW 264.7 cells and probed with the anti-Thr(P)<sup>294</sup> antibody. The MEKK3 band was immunoreactive with the Thr(P)<sup>294</sup> antibody, suggesting that endogenous MEKK3 was phosphorylated at Thr<sup>294</sup> (Fig. 5B). Western blotting was performed using the Odyssey infrared scanner, and therefore we were able to directly quantify the level of total MEKK3 (800-nm channel) and Thr(P)<sup>294</sup> (700-nm channel) on the same immunoblots (two-color Western blotting). This provided us with a highly accurate method for quantifying the level of Thr<sup>294</sup> phosphorylation of endogenous MEKK3. The ratio of Thr(P)<sup>294</sup> to total MEKK3 significantly decreased with calf intestinal phosphatase (CIP) treatment (Fig. 5B), suggesting that endogenous MEKK3 is phosphorylated at Thr<sup>294</sup>. The residual Thr(P)<sup>294</sup> signal could represent either incomplete dephosphorylation by CIP or a low amount of antibody reactivity with nonphosphorylated MEKK3.

Next, we treated RAW 264.7 cells with TNFα, which stimulates MEKK3 through activation TNF receptor 1. The cells were placed in fresh medium for 4 h (to remove secreted TNFα) and then stimulated with TNFα. Again, these experiments utilized two-color infrared Western blotting, and quantification of the Thr(P)<sup>294</sup> signal to total MEKK3 revealed a decrease in phosphorylation, with a maximal decrease in Thr<sup>294</sup> signal of 47% following 20 min of stimulation (Fig. 5C). A time course experiment showed that the reduction in Thr<sup>294</sup> phosphorylation was transient following TNFα stimulation and had almost returned to unstimulated levels by 60 min (Fig. 5D).

We also tested the Toll-like receptor 4 agonist LPS, which stimulates numerous signaling pathways including MEKK3 (17). LPS treatment also caused a reduction of Thr<sup>294</sup> phosphorylation by 61% within 30 min (Fig. 5E). The decrease in phosphorylation of Thr<sup>294</sup> occurred with similar kinetics compared with ERK1 and 2 phosphorylation (Fig. 5E).

Endogenous 14-3-3-MEKK3 Interaction Is Disrupted by LPS—Next, we asked whether the dephosphorylation of Thr<sup>294</sup> might affect the association of endogenous 14-3-3 with endogenous MEKK3. We used LPS stimulation for these experiments, because LPS caused the greatest apparent decrease in Thr<sup>294</sup> phosphorylation. Following LPS treatment, co-immunoprecipitation of 14-3-3 was abolished, in parallel with the decrease in Thr<sup>294</sup> phosphorylation (Fig. 6A). We reasoned that the lower amount of residual Thr<sup>294</sup> phosphorylation following LPS stimulation was insufficient to maintain 14-3-3 binding. Alternatively, as we speculate above based on the CIP experiment in Fig. 5, the anti-Thr(P)<sup>294</sup> antibody could be reacting to some extent with nonphosphorylated MEKK3. Thus, to confirm that 14-3-3 was binding to endogenous MEKK3 at Thr<sup>294</sup>, we conducted peptide competition assays similar to Fig. 4. Incubation with the phospho-peptide corresponding to Thr<sup>294</sup> disrupted the interaction of endogenous MEKK3 and endogenous 14-3-3, but not a nonphospho-peptide or a scrambled
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FIGURE 5. Endogenous MEKK3 is phosphorylated at Thr$^{294}$ and is reduced by LPS and TNF$\alpha$ treatment. A, RAW 264.7 macrophage cells were solubilized, and lysates were exposed to three commercially available MEKK3 antibodies: 5 µg of BD Biosciences 611102 mouse monoclonal, 5 µg of Epitomics 1672 rabbit monoclonal, or 5 µg of Epitomics 1673 rabbit monoclonal. Antibody-MEKK3 complexes were immunoprecipitated (IP) and immunoblotted with anti-MEKK3 (BD 611102). Both the BD 611102 and Epitomics 1673, but not Epitomics 1672, immunoprecipitated endogenous MEKK3. This result was repeated three times with similar results. B, RAW 264.7 cells (10$^6$) were solubilized, and MEKK3 was immunoprecipitated with BD 611102. The immunoprecipitates were washed with solubilization buffer and then washed twice with CIP buffer. CIP (0.1 µg) was added to one of the tubes, and all of the tubes were incubated for 10 min at 37 °C. The beads were washed once with solubilization buffer and then boiled in sample buffer. The proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was incubated with anti-MEKK3 antibody (BD 611102) and anti-Thr(P)$^{294}$ antibody simultaneously and then decorated with anti-mouse IRDye 800 and anti-mouse IRDye 700 secondary antibodies (LiCor). Detection was performed using a LiCor Odyssey infrared scanner. Quantification of the MEKK3 and Thr(P)$^{294}$ bands was performed using LiCor Odyssey 2.1 Software. The merge image is the overlay of the 700 nm (green), which represents total MEKK3) channels and the 800 nm (red), which represents Thr(P)$^{294}$) and 800 nm (green), which represents total MEKK3) channels. The numbers beneath each band represent the ratio of Thr(P)$^{294}$ signal to MEKK3 signal. This experiment was repeated twice with similar results. C, RAW 264.7 cells were placed in fresh medium for 4 h and then stimulated for 20' with TNF$\alpha$ (5 ng/ml). MEKK3 was immunoprecipitated, and Thr(P)$^{294}$ levels were detected as describe in 8 above. Mock IP represents lysates receiving beads alone. This experiment is representative of three independent experiments. D, RAW 264.7 cells were placed in fresh medium for 4 h and then stimulated in duplicate for the indicated times with TNF$\alpha$ (5 ng/ml) where indicated. MEKK3 was immunoprecipitated and Thr(P)$^{294}$ levels were detected as described in 8 above. Similar results were observed in two independent experiments performed in duplicate. E, RAW 264.7 cells were treated with LPS (1 µg/ml) for the indicated times. Endogenous MEKK3 was immunoprecipitated with BD 611102, and Thr(P)$^{294}$ levels were detected as described in 8 above. To verify that LPS was activating the TLR4 in these cells, we monitored the phosphorylation of ERK1 and 2 using a phosphospecific antibody specific for activated ERK. Induction of ERK phosphorylation paralleled the decrease in MEKK3 Thr$^{294}$ phosphorylation. Similar results were observed in three independent experiments with LPS.

 phospho-peptide control (Fig. 6B). Together these results show that endogenous 14-3-3 interacts with endogenous MEKK3 at Thr(P)$^{294}$, and this is disrupted upon LPS-stimulated reduction of Thr$^{294}$ phosphorylation.

In this same set of experiments, we asked whether phosphorylation of Ser$^{526}$ of endogenous MEKK3 was altered by LPS treatment. Previous work by Zhang et al. (16) showed that Ser$^{526}$ phosphorylation increases upon LPS treatment. Probing with our anti-Ser(P)$^{526}$ antibody showed that Ser$^{526}$ phosphorylation was elevated by about 60% following LPS stimulation (Fig. 6C). As before, these experiments utilized the LiCor Odyssey infrared scanner, and the ratio of Ser(P)$^{526}$ signal to total endogenous MEKK3 was analyzed by two-color Western blotting on the same MEKK3 band. Probing with anti-14-3-3 showed that the loss of 14-3-3 binding correlated with the increase in Ser$^{526}$ phosphorylation (Fig. 6C).

T294A MEKK3 Elevates NF-κB Response to TNF$\alpha$—Taken together, our results with endogenous proteins demonstrate that MEKK3 is constitutively phosphorylated at Thr$^{294}$, and this phosphorylation in reduced by TNF$\alpha$ or LPS stimulation. The reduction in Thr$^{294}$ phosphorylation promoted the release of endogenous 14-3-3 interaction. Finally, the loss of 14-3-3 binding correlated with a small increase in activation loop phosphorylation at Ser$^{526}$ for endogenous MEKK3. We therefore hypothesized that the loss of 14-3-3 binding could be involved in stimulation of MEKK3 signaling.

When MEKK3 was expressed in cells, we found that both Thr$^{294}$ and Ser$^{526}$ phosphorylation were phosphorylated to a high level and were not altered significantly by either TNF$\alpha$ or LPS stimulation. This is different from endogenous MEKK3, where the levels of MEKK3 and the degree of regulation are likely to experience finer control. To evaluate how phosphorylation of Thr$^{294}$ and binding to 14-3-3 might modulate MEKK3 signaling in cells, we first performed in vitro kinase assays comparing WT MEKK3, T294A MEKK3, and K391M MEKK3. As expected, the kinase dead K391M MEKK3 did not undergo autophosphorylation (Fig. 7A). Both the WT MEKK3 and T294A MEKK3 experienced very similar levels of autophosphorylation (Fig. 7A). This
result shows that T294A MEKK3 does not significantly perturb the intrinsic activity of MEKK3.

We next performed NF-κB reporter experiments to monitor the activation of NF-κB in cells expressing WT MEKK3 or T294A MEKK3 following exposure to TNFα. We used the luciferase reporter assay to provide quantifiable measurements that could distinguish small changes in pathway activation. Luciferase activity was low in cells expressing WT MEKK3 or T294A MEKK3 without stimulation but was stimulated by 4 h of TNFα treatment (Fig. 7B). TNFα increased the level of T294A MEKK3-mediated NF-κB activation significantly more than WT MEKK3 (Fig. 7B). The luciferase measurements were normalized to an internal β-galactosidase control, and the level of expression of MEKK3 was identical between WT and T294A MEKK3 (Fig. 7B). Thus, expression of the T294A MEKK3 elevated TNFα-stimulated NF-κB activation compared with WT MEKK3.

DISCUSSION

The MEKK2 and MEKK3 protein kinases play important roles in the activation of numerous MAPK and NF-κB signaling pathways following cellular stress and activation by pro-inflamatory cytokines. For example, MEKK3 is essential for inflammatory gene expression induced downstream of TNF receptor-1, IL-1 receptor and Toll-like receptor activation through the activation of c-Jun N-terminal kinase, p38 MAPK, and NF-κB (1, 17). The mechanisms that control MEKK2 and MEKK3 activation and their target specificity are thus a subject of intense interest.
Specificity of signaling is coordinated through hierarchical phosphorylation cascades that are regulated through association between signaling proteins. The well known phosphoprotein-binding molecule 14-3-3 plays many roles in controlling the specificity, signal strength, and spatial localization of numerous pathways (18). 14-3-3 has been shown to interact with MEKK3, but the site of interaction has not yet been determined. We performed tryptic mapping, phosphoamino acid analysis, and immunoblot analysis with a phosphospecific antibody to identify Thr\(^{294}\) as a novel site of MEKK3 phosphorylation. Mutation of this residue to alanine abolished 14-3-3 interaction, as did incubation of MEKK3 with a synthetic peptide containing phosphorylated but not the unphosphorylated Thr\(^{294}\) peptide or a scrambled phosphorylated peptide.

Phosphorylation of Thr\(^{294}\) was dependent upon the catalytic activity of MEKK3, because mutation of the catalytic lysine at 391 significantly diminished phosphorylation of Thr\(^{294}\) and dramatically reduced the interaction between MEKK3 and 14-3-3. This suggested that phosphorylation of Thr\(^{294}\) occurred by autophosphorylation. However, it is also possible that a downstream kinase activated by MEKK3 phosphorylated Thr\(^{294}\). For endogenous MEKK3, Thr\(^{294}\) appeared to be constitutively phosphorylated and was reduced upon stimulation with TNFα and LPS. The residual phosphorylation of endogenous MEKK3 detected by the phospho-specific Thr\(^{294}\) antibody could be cross-reactivity to nonphosphorylated MEKK3, which is supported by the CIP experiment shown in Fig. 5. If this is the case, then the actual decrease in Thr\(^{294}\) phosphorylation of endogenous MEKK3 could be higher than our analysis suggests, in agreement with the observation that 14-3-3 binding is entirely lost upon LPS stimulation. Alternatively, reduced Thr\(^{294}\) phosphorylation could be sufficient to disengage 14-3-3 from endogenous MEKK3. Importantly, phosphopeptides mimicking Thr(P)\(^{294}\) competed with endogenous MEKK3 for 14-3-3 interaction.

We found that in cells overexpressing MEKK3, Ser\(^{526}\) phosphorylation was similar between WT MEKK3 and T294A MEKK3. However, when we excluded okadaic acid from the solubilization buffer, we noticed that WT and T294A MEKK3 experienced a significant reduction of Ser\(^{526}\) phosphorylation. These observations are consistent with the recent report that Ser\(^{526}\) is sensitive to PP1/2A phosphatase activity in cells (15). These observations are consistent with the recent report that Ser\(^{526}\) is sensitive to PP1/2A phosphatase activity in cells (15). We performed tryptic mapping, phosphoamino acid analysis, and immunoblot analysis with a phosphospecific antibody to identify Thr\(^{294}\) as a novel site of MEKK3 phosphorylation. Mutation of this residue to alanine abolished 14-3-3 interaction, as did incubation of MEKK3 with a synthetic peptide containing phosphorylated but not the unphosphorylated Thr\(^{294}\) peptide or a scrambled phosphorylated peptide.

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We found that in cells overexpressing MEKK3, Ser\(^{526}\) phosphorylation was similar between WT MEKK3 and T294A MEKK3. However, when we excluded okadaic acid from the solubilization buffer, we noticed that WT and T294A MEKK3 experienced a significant reduction of Ser\(^{526}\) phosphorylation. These observations are consistent with the recent report that Ser\(^{526}\) is sensitive to PP1/2A phosphatase activity in cells (15). However, in our experiments we could not discriminate a difference between WT and T294A MEKK3 in the sensitivity to dephosphorylation at Ser\(^{526}\).

Interestingly, when we monitored Ser\(^{526}\) phosphorylation of endogenous MEKK3, we noted a small increase in Ser\(^{526}\) phosphorylation following stimulation with LPS, which was consistent with the work of Zhang et al. (16), who initially described the identity of this site. Thus, our work is in agreement with previous evidence that Ser\(^{526}\) phosphorylation increases upon LPS stimulation, and this increase parallels the drop in Thr\(^{294}\) phosphorylation and the loss of 14-3-3 binding. This suggests that dephosphorylation of MEKK3 at Thr\(^{294}\) and the subsequent loss of 14-3-3 binding might contribute to agonist-induced phosphorylation of Ser\(^{526}\) and could be one mechanism that might account for our observation that T294A MEKK3 stimulated more TNFα-induced NF-κB activity compared with WT MEKK3.

The closely related MEKK2 protein kinase shares significant homology to MEKK3 but might have distinct signaling functions and may participate in the sustained activation of NF-κB by regulating the activation of IKK and degradation of IκBβ (2). MEKK2 is highly homologous to MEKK3 in amino acid sequence surrounding the equivalent site of Thr\(^{294}\), suggesting that MEKK2 might associate with 14-3-3 in a similar manner to MEKK3. This suggested that phosphorylation of Thr\(^{283}\)/Thr\(^{294}\) is novel and unique to the MEKK2/3 family. The discovery of this novel site and role in mediating MEKK3 activity provides an important clue in how the pro-inflammatory cytokine TNFα and the endotoxin LPS regulate signals to control inflammatory gene expression.

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