Phosphorylation of Thr-516 and Ser-520 in the Kinase Activation Loop of MEKK3 Is Required for Lysophosphatidic Acid-mediated Optimal IκB Kinase β (IKKβ)/Nuclear Factor-κB (NF-κB) Activation*

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MEKK3 serves as a critical intermediate signaling molecule in lysophosphatidic acid-mediated nuclear factor-κB (NF-κB) activation. However, the precise regulation for MEKK3 activation at the molecular level is still not fully understood. Here we report the identification of two regulatory phosphorylation sites at Thr-516 and Ser-520 within the kinase activation loop that is essential for MEKK3-mediated IκB kinase β (IKKβ)/NF-κB activation. Substitution of these two residues with alanine abolished the ability of MEKK3 to activate IKKβ/NF-κB, whereas replacement with acidic residues rendered MEKK3 constitutively active. Furthermore, substitution of these two residues with alanine abolished the ability of MEKK3 to mediate lysophosphatidic acid-induced optimal IKKβ/NF-κB activation.

Transcription factor nuclear factor-κB (NF-κB) plays an important role in immunity, stress responses, anti-apoptosis, cell proliferation, and differentiation (1–4). In unstimulated cells, NF-κB is sequestered in the cytoplasm by members of the inhibitors of NF-κB (IκB) family acting as inhibitory proteins. Following cell stimulation, IκB proteins are phosphorylated by activated IκB kinase (IKK) (5–12). This phosphorylation event induces IκB protein ubiquitination and degradation (1, 13, 14). Degradation of the IκB proteins liberates NF-κB and allows its translocation to the nucleus, where it controls the expression of the target genes (15).

Lysophosphatidic acid (LPA), a naturally occurring water-soluble glycerophospholipid, controls many cellular responses such as cell proliferation, chemotaxis, and survival through binding to its cognate G protein-coupled receptors and activating LPA receptor-mediated intracellular signaling pathways, including IKKβ/NF-κB (16). Several adaptor and scaffold proteins, such as β-arrestin2, Bcl10 (B-cell CLL/lymphoma 10), MALT1 (mucosa-associated lymphoid tissue lymphoma translocation gene 1), and CARMA3 (CARD and MAGUK domain-containing protein 3), were identified as essential components of intermediate signaling molecules to mediate LPA-induced NF-κB activation (17–21). Recently, MEKK3 (mitogen-activated protein kinase kinase kinase 3), but not TAK1 (transforming growth factor-β-activated kinase 1), has been demonstrated to play an essential role in LPA- and protein kinase C (PKC)-induced IKKβ/NF-κB activation in mouse embryonic fibroblast (MEF) cells (22).

One of the critical steps for kinase activation is phosphorylation of the specific serine or threonine residues within the activation loop of the protein kinase located between kinase subdomains VII and VIII (23). In the case of MEKK3, several studies have been carried out to identify the phosphorylation sites and the effect of phosphorylation on MEKK3 activity. For example, phosphorylation of Ser-166 and Ser-337 has been shown to be promoted by tumor necrosis factor α but not required for MEKK3 activity (24), and phosphorylation of Ser-526 is required for MEKK3-mediated MAPK and NF-κB activation (25, 26). However, the molecular mechanism of MEKK3-mediated IKKβ/NF-κB activation remains to be clearly defined.

In this study, we further examine the potential phosphorylation sites in the kinase activation loop of MEKK3 that is required for MEKK3-mediated NF-κB activation. Using mutational analysis and reporter assays, we identified Thr-516 and Ser-520 within kinase subdomains VII and VIII as two additional regulatory phosphorylation sites required for MEKK3-mediated IKKβ/NF-κB activation. We demonstrate that both Thr-516 and Ser-520 on MEKK3 are phosphorylated by using specific antibodies that recognize the phosphorylation of these two sites. Phosphorylation of these two residues on MEKK3 is required for LPA-induced optimal IKKβ/NF-κB activation as well as interleukin-6 (IL-6) production.
Mechanism of MEKK3-induced NF-κB Activation

EXPERIMENTAL PROCEDURES

Plasmid Constructs—A cDNA construct containing the full-length open reading frame of wild-type human MEKK3 was subcloned into the hemagglutinin (HA)-tagged mammalian expression vector pcDNA3.1. The following mutant MEKK3 expression constructs were generated by site-directed PCR mutagenesis and verified by DNA sequencing: K391R, S511A, T516A, S520A, T522A, S526A, T516A/S520A, T516E/S520A, T516A/S520D, and T516E/S520D. The NF-κB-dependent firefly luciferase reporter plasmid and pCMV promoter-dependent Renilla luciferase reporter plasmid were purchased from Clontech (Mountain View, CA). The retroviral expression vectors were constructed by subcloning the empty vector, HA-MEKK3-wild type (WT), and HA-MEKK3-T516A/S520A cDNA into the pBabe vector.

Antibodies and Reagents—Antibodies against phospho-IKKα/β (catalogue no. 2681), IKKβ (2684), phospho-JNK (9251), JNK (9252), phospho-ERK1/2 (9106), ERK1/2 (9102), phospho-IkBα (9246), and IkBα (9242) and secondary antibodies conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against HA epitope (F-7), proliferating cell nuclear antigen (PC-10), and NF-κB-p65 (F-6) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against MEKK3 (611103) was from BD Biosciences. Antibody against β-actin was from Sigma. Polyclonal antibody specific for human phospho-MEKK3 (pThr-516/pSer-520) was produced by immunizing rabbits with MEKK3 phosphopeptide (GASKRLQpTIC-MpSGTGMR) at Genemed Synthesis, Inc. (San Antonio, TX). Protein phosphatase 1 (PP1) and λ-protein phosphatase (λ-PPase) were purchased from New England Biolabs (Ipswich, MA). FuGENE 6 transfection reagent was from Roche Applied Science. Lipofectamine 2000 transfection reagent was from Invitrogen. LPA, phorbol 12-myristate 13-acetate (PMA), ionomycin (Iono), and Polybrene were purchased from Sigma. The mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kit was purchased from BD Biosciences. The ECL-Plus Western blotting system was purchased from GE Healthcare.

Cell Culture and Transfection—HEK-293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and transfected with FuGENE 6 according to the manufacturer’s recommendations. The above medium also contained penicillin (100 units/ml), streptomycin (100 mg/ml), and glutamine (2 mm). MEKK3-deficient (MEKK3<sup>−/−</sup>) MEFs were generated in Dr. Bing Su’s laboratory, cultured as described previously (27), and transfected with Lipofectamine 2000 following the manufacturer’s instructions.

Establishment of Stable MEKK3<sup>−/−</sup> MEF Cells Lines Expressing Empty Vector, HA-MEKK3-WT, and HA-MEKK3-T516A/S520A—The retroviral supernatants were generated in HEK-293T cells as described (28). In brief, the pBabe-HA-MEKK3-WT, pBabe-HA-MEKK3-T516A/S520A, or pBabe empty vector was co-transfected into HEK-293T cells with a pCL-Eco vector using FuGENE 6 transfection reagent according to the manufacturer’s instructions. Viral supernatants were collected after 36 and 48 h. MEKK3<sup>−/−</sup> MEF cells were incubated with virus-containing medium in the presence of 4 µg/ml Polybrene. Stable cell lines were established after 5 days of puromycin (3 µg/ml) selection.

Luciferase Reporter Gene Assay—Luciferase reporter gene assay was performed using a Dual-Luciferase reporter assay system (Promega, Madison, WI) and a Monolight 3010 luminometer (Pharmingen) as described previously (28). Briefly, transfected cells were transiently cotransfected with specific vectors and an NF-κB-dependent firefly luciferase reporter construct as well as a Renilla luciferase control construct. Cellular extracts were prepared 36 h post-transfection, and the luciferase activities were determined. Relative NF-κB luciferase activity was normalized to Renilla luciferase activity. Changes in luciferase activity with respect to control were calculated. Each experiment was conducted in triplicate.

Preparation of Nuclear and Cytosolic Fractions—Nuclear and cytosolic extracts were made as described (27). In brief, cells were harvested in ice-cold phosphate-buffered saline (pH 7.4) and pelleted by centrifugation at 500 × g for 3 min and then lysed for 30 min on ice in buffer A (10 mM HEPES buffer (pH 7.9) containing 0.1 mM EDTA, 10 mM KCl, 0.4% (v/v) IGEPAL, 0.5 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 15,000 × g for 10 min. The resulting supernatants constituted cytosolic fractions. The pellets were washed three times with buffer A and resuspended in buffer B (20 mM HEPES buffer (pH 7.9) containing 400 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 1 mM phenylmethylsulfonyl fluoride), incubated for 30 min on ice, and centrifuged at 15,000 × g for 10 min. The supernatants were used as nuclear extracts.

Immunoblotting and Immunoprecipitation—Cells were harvested in ice-cold phosphate-buffered saline (pH 7.4) and spun down. The pellet was dissolved in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% IGEPAL, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM benzamidine, 20 mM disodium p-nitrophenyl phosphate, 0.1 mM sodium orthovanadate, 10 mM sodium fluoride, and phosphatase inhibitor mixture A and B (Sigma)). The cell lysates were either subjected directly to 10% SDS-PAGE for immunoblotting analysis or immunoprecipitated 3 h with the indicated antibodies. Immune complexes were recovered with Protein A-agarose (Santa Cruz Biotechnology) for 3 h and then washed three times with wash buffer containing 20 mM HEPES (pH 7.4), 50 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, and 0.05% Triton X-100. For immunoblotting, the immunoprecipitates or whole cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL-Plus Western blotting system according to the manufacturer’s instructions.

In Vitro Dephosphorylation Assay of Phospho-MEKK3 by PP1 or λ-PPase—HEK-293T cells were transfected with a HA-MEKK3 expression plasmid or an empty vector. Thirty-six hours after transfection, cell extracts were prepared in buffer

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containing 50 mM Tris-HCl (pH 7.4) 150 mM NaCl, 1 mM EDTA, 1% IGEPA, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM benzamidine. The HA-tagged MEKK3 protein was immunoprecipitated 3 h with anti-HA antibody-conjugated Protein A-agarose and washed three times with lysis buffer and twice with phosphatase reaction buffer (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.1 mM EGTA, 0.025% Tween 20, 1 mM MnCl₂ (pH 7.5)). One unit of PP1 or λ-PPase with phosphatase reaction buffer was then added into the tubes containing the precipitated MEKK3 protein incubated for 25 min at 30 °C. Laemmli sample buffer was added to the reaction and boiled at 95 °C for 5 min, and the proteins were resolved by SDS-PAGE and then immunoblotted with anti-HA antibodies.

**Electrophoretic Mobility Shift Assay**—NF-κB oligonucleotide probes were labeled with γ32P[ATP. MEF cells (1 × 10⁴) were starved for 12 h and stimulated for the indicated time points. Nuclear extracts isolated from these cells were then incubated with immunoblotting (IB). β-Actin was detected as a loading control. C, MEKK3-T516E/S520D acts as a constitutively active mutant to induce NF-κB activation. One microgram of NF-κB luciferase reporter plasmid and 20 ng of Renilla luciferase plasmid were cotransfected into HEK-293T cells transfected with expression vectors for HA-MEKK3 and its derived mutants, as indicated, and analyzed by immunoblotting with anti-MEKK3 (pThr-516/pSer-520) antibodies (top panel) and anti-HA antibodies (bottom panel). B, anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies detect only phosphorylated MEKK3. Cell extracts prepared from HEK-293T cells transfected with an expression vector for HA-MEKK3 were immunoprecipitated with anti-HA antibodies and then treated with or without PP1 or λ-PPase for 30 min before being analyzed by immunoblotting (IB) with anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies (top panel) or anti-HA antibodies (bottom panel). C, the anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies recognize LPA-induced MEKK3 phosphorylation. MEKK3−/− MEF cells reconstituted with HA-MEKK3 were untreated or treated with LPA (30 μM) for 0, 3, 5, and 7 min and harvested. Cell extracts were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies. Total HA-MEKK3 proteins present in the immunoprecipitates were detected by stripping the blots and reprobing with anti-HA antibodies.

**FIGURE 2.** Generation and characterization of antibodies specific for phospho-MEKK3 at Thr-516 and Ser-520. A, anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies are specific for human MEKK3 pThr-16/pSer-20. Cell extracts were prepared from HEK-293T cells transfected with expression vectors for HA-tagged full-length MEKK3 and its derived mutants, as indicated, and analyzed by phospho-MEKK3 (pThr-516/pSer-520) antibodies (top panel) and anti-HA antibodies (bottom panel). B, anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies detect only phosphorylated MEKK3. Cell extracts prepared from HEK-293T cells transfected with an expression vector for HA-MEKK3 were immunoprecipitated with anti-HA antibodies and then treated with or without PP1 or λ-PPase for 30 min before being analyzed by immunoblotting (IB) with anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies (top panel) or anti-HA antibodies (bottom panel). C, the anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies recognize LPA-induced MEKK3 phosphorylation. MEKK3−/− MEF cells reconstituted with HA-MEKK3 were untreated or treated with LPA (30 μM) for 0, 3, 5, and 7 min and harvested. Cell extracts were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies. Total HA-MEKK3 proteins present in the immunoprecipitates were detected by stripping the blots and reprobing with anti-HA antibodies.

**FIGURE 1.** Phosphorylation of both Thr-516 and Ser-520 at the activation loop of human MEKK3 protein is required for MEKK3-mediated NF-κB activation. A, sequence alignment of the MEKK3 kinase activation loop from different species. Potential phosphorylation sites are marked with an open box. The known phosphorylation site of MEKK3 Ser-526 is marked with an asterisk. B, effect of alanine substitution at the indicated residue within the activation loop on MEKK3-induced NF-κB activation. One microgram of NF-κB luciferase (Luc) reporter plasmid and 20 ng of Renilla luciferase plasmid were cotransfected into HEK-293T cells with 1 μg of empty control vector or MEKK3 expression vectors for HA-MEKK3 and its derived mutants, as indicated, and analyzed by phospho-MEKK3 (pThr-516/pSer-520) antibodies. Total HA-MEKK3 proteins were immunoprecipitated with anti-HA antibodies and then treated with or without PP1 or λ-PPase as indicated, and analyzed by immunoblotting (IB) with anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies. NF-κB activity was measured 36 h later and normalized with α-tubulin activity. Error bars indicate ± S.D. in triplicate experiments. Expression levels of different HA-MEKK3 constructs in the transfected cells were detected by immunoblotting (IB). β-Actin was detected as a loading control.
Mechanism of MEKK3-induced NF-κB Activation

A. MEKK3+/– MEF cells

HA-MEKK3 HA-IKKβ IP: HA

Stripped: HA WCL

HA-MEKK3 HA-IKKβ p-IKKβ

B. MEKK3+/– MEF cells

NF-κB-Luc

Relative Luciferase Activity

HA-MEKK3 Wild type T516E/S520A T516K T516A/S520A K391R T516A/S520A T516E/S520A

IB: HA-MEKK3

IB: β-actin

C. MEKK3+/– MEF cells

NF-κB-Luc

Relative Luciferase Activity

HA-MEKK3 Wild type T516E/S520A T516K T516A/S520A K391R T516A/S520A T516E/S520A

IB: HA-MEKK3

IB: β-actin

FIGURE 3. Phosphorylation of both Thr-516 and Ser-520 is required for MEKK3-mediated IKK/NF-κB activation. A, phosphorylation of both Thr-516 and Ser-520 on MEKK3 is essential for MEKK3-mediated phosphorylation of IKKβ. MEKk3+/– MEF cells were cotransfected with empty vector, HA-MEKK3-WT expression vector, or derived mutants in the presence of HA-IKKβ using Lipofectamine 2000. After 36 h, cell extracts were immunoprecipitated (IP) with anti-HA antibodies and immunoblotted (IB) with anti-phospho-IKKβ antibodies; proteins present in the immunoprecipitates were detected by stripping the blots and reprobing with anti-HA antibodies. The expression level of HA-MEKK3-WT and derived mutant proteins was detected by anti-MEKK3 antibodies. The level of β-actin expression probed by anti-β-actin antibodies was detected as a loading control. B, phosphorylation of both Thr-516 and Ser-520 is required for MEKK3-induced NF-κB luciferase (Luc) activity. Cell Proliferation Assay—Empty vector-, MEKK3-WT-, and MEKK3-AA-reconstituted MEF cells were seeded on 96-well plates with 1 × 10⁴ cells/well. Twenty-four hours later, cells were treated with or without LPA (30 μM) or PMA (40 ng/ml) plus Iono (100 ng/ml). Cell numbers in each well were counted after treatment for 24, 48, and 72 h. Each experiment was conducted in triplicate.

RESULTS

Thr-516 and Ser-520 within the Activation Loop of Human MEKK3 Are Two Potential Phosphorylation Sites Essential for MEKK3-mediated NF-κB Activation—Phosphorylation of serine and/or threonine residue(s) within kinase subdomains VII and VIII is essential for the activation of many kinases involved in the regulation of NF-κB activation such as IKK (23). We aligned amino acid sequences of the MEKK3 kinase activation loop from various species and indicated five conserved Ser/Thr in the activation loop (Fig. 1A). To identify other potential phosphorylation sites on MEKK3 that are essential for its NF-κB activation, an NF-κB-dependent luciferase reporter assay was chosen to screen the effect of point mutants of serine/threonine residues within the activation loop of MEKK3 on its NF-κB activation. We systematically mutated five serine and threonine residues to alanine within the kinase activation loop between kinase subdomains VII and VIII of human MEKK3 to examine whether these mutations would affect MEKK3-induced NF-κB activation in a reporter assay (Fig. 1B). The expression plasmid encoding wild-type MEKK3, the active site lysine-to-arginine mutant (K391R), or each point mutant of MEKK3 was transfected into HEK-293T cells, along with plasmids containing an NF-κB-dependent luciferase reporter gene. As shown in Fig. 1B, three MEKK3 mutations (T516A, S520A, and S526A) resulted in the inhibition of MEKK3-induced NF-κB luciferase reporter gene expression in the cells. These results suggest that Thr-516 and Ser-520, in addition to Ser-526, are also potential phosphorylation sites essential for MEKK3-mediated NF-κB activation.

Because Ser-526 has been reported to be essential for MEKK3-mediated MAPK and NF-κB activation (25, 26), we hypothesized that Thr-516 and Ser-520 are two potential phosphorylation sites essential for MEKK3-mediated NF-κB activation. To identify other potential phosphorylation sites on MEKK3, we performed a reporter assay in triplicate experiments. Expression levels of different HA-MEKK3 constructs in transfected cells were detected by immunoblotting. β-Actin was detected as a loading control. WCL, whole cell lysates.
tributing to MEKK3-mediated NF-κB activation. To test this hypothesis, we made substitutions of Thr-516 and Ser-520 with single or double acidic residues (MEKK3-T516E and MEKK3-S520D) to mimic the phosphorylation of these two residues. As shown in Fig. 1C, MEKK3-T516E/S520D double mutants induced NF-κB activation in the reporter assay at a much higher level compared with wild-type MEKK3 or the T516E or S520D single mutant, whereas the T516E/S520A, T516A/S520D, and T516A/T520A double mutants failed to activate the NF-κB reporter gene. These results strongly suggest that the MEKK3-T516E/S520D mutant is constitutively active in the assay and that phosphorylation of both Thr-516 and Ser-520 is required for the MEKK3-mediated NF-κB activation.

Successful Generation of Anti-phospho-MEKK3 (pThr-516/pSer-520) Antibodies, and LPA Induces MEKK3 Phosphorylation at Thr-516 and Ser-520—To examine directly whether Thr-516 and Ser-520 of human MEKK3 are phosphorylated...
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**Figure 5. Phosphorylation of both Thr-516 and Ser-520 on MEKK3 is required for PKC-induced IL-6 production and cell proliferation.**

A and B, phosphorylation of Thr-516 and Ser-520 on MEKK3 is required for LPA- and PKC-induced IL-6 production. MEKK3^-/- MEF cells reconstituted with empty vector, HA-MEKK3-WT, and HA-MEKK3-AA were untreated or treated with LPA (A) or PMA/Iono (P/I) (B) for the time points indicated. The supernatants from these cultures were collected and subjected to mouse IL-6 (mIL-6) ELISA analysis according to the manufacturer’s instructions. C and D, phosphorylation of Thr-516 and Ser-520 on MEKK3 is required for LPA- and PKC-induced cell proliferation. MEKK3^-/- MEF cells reconstituted with empty vector, HA-MEKK3-WT, and HA-MEKK3-AA were untreated or treated with LPA (C) or PMA/Iono (D) for the time points indicated, and then the cell numbers were counted.

During its activation, rabbit antibodies specifically recognizing Thr-516- and Ser-520-phosphorylated human MEKK3 were generated by immunizing rabbits with a synthetic phosphopeptide (keyhole limpet hemocyanin-coupled) corresponding to residues surrounding Thr-516 and Ser-520 of human MEKK3. Antibodies were purified by Protein A-agarose and peptide affinity chromatography. As shown in Fig. 2A, our antibodies specific for phospho-MEKK3 (pThr-516/pSer-520) strongly recognized wild-type MEKK3 but not T516A/S520A and T516E/S520D double mutant proteins. Furthermore, our antibodies also weakly recognized MEKK3-T516A and MEKK3-S520A single mutant proteins. To further validate the specificity of our antibodies, a mammalian expression vector encoding wild-type HA-tagged MEKK3 was transfected into HEK-293T cells. Overexpressed MEKK3 proteins were immunoprecipitated from cell lysates and treated with PP1 or H9261-PPase and resolved by 10% SDS-PAGE. As shown in Fig. 2B, our antibodies recognized only the nontreated but not the phosphatase-treated wild-type MEKK3, although the same amount of MEKK3 protein was loaded. These results demonstrate that our antibodies specifically detect MEKK3 only when phosphorylated at Thr-516 and/or Ser-520.

MEKK3 is essential for LPA-mediated IKK/ NF-κB activation (22). To test whether LPA could induce MEKK3 phosphorylation on Thr-516/Ser-520, we stimulated HA-MEKK3-reconstituted Mekk3^-/- MEF cells with LPA for 0, 3, 5, and 7 min, respectively (Fig. 2C). HA-MEKK3 proteins in the cell lysates were immunoprecipitated with anti-HA antibodies and then immunoblotted with anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies. In this assay, we found that LPA induced MEKK3 phosphorylation on Thr-516/Ser-520 (Fig. 2C). These data indicate that MEKK3 Thr-516 and Ser-520 phosphorylation is essential for MEKK3 activation.

Phosphorylation of Thr-516 and Ser-520 Is Required for MEKK3-mediated IKK/NF-κB Activation—Phosphorylation of IKKβ Ser-177 and Ser-181 in the kinase activation loop is essential for IKK activation (23). Overexpression of MEKK3 results in IKK activation (27, 29). To test whether phosphorylation of both Thr-516 and Ser-520 sites is required for MEKK3-mediated IKK phosphorylation at Ser-177 and Ser-181, we transfected the full-length wild-type and mutant MEKK3 expression plasmids with HA-tagged IKKβ into Mekk3^-/- MEF cells. The HA-tagged IKKβ proteins were immunoprecipitated from the cell lysates with anti-HA antibodies and immunoblotted with antibodies specific for phospho-IKKα/β. As shown in Fig. 3A, overexpression of only MEKK3-WT and the MEKK3-T516E/S520D mutant resulted in IKKβ phosphorylation at Ser-177 and Ser-181, whereas the MEKK3-T516E/S520D mutant induced a higher level of IKKβ phosphorylation compared with MEKK3-WT. Other mutants were not able to induce the visible IKKβ phosphorylation at all. Consistent with these results, luciferase analysis with an NF-κB-responsive reporter showed that only wild-type and T516E/S520D mutant MEKK3 significantly induced the activity of NF-κB in Mekk3^-/- MEF cells (Fig. 3B).
Previous studies demonstrate that MEKK3 Ser-526 is essential for MEKK3-mediated NF-κB activation (25, 26). Interestingly, we found the MEKK3-T516E/S520D/S526A mutant failed to induce NF-κB reporter activity compared with MEKK3-WT and the MEKK3-T516E/S520D mutant (Fig. 3C). This result indicates that MEKK3 phosphorylation at Thr-516/Ser-526 is essential for MEKK3-mediated NF-κB activation.

Taken together, our results suggest that phosphorylation of Thr-516 and Ser-520 sites is required for MEKK3-mediated IKKβ/NF-κB activation. Once overexpressed, the MEKK3-T516E/S520D mutant acts as a constitutively active mutant to induce IKKβ/NF-κB activation.

Phosphorylation of Thr-516 and Ser-520 on MEKK3 Is Required for LPA- and PKC-induced IKK/NF-κB Activation—MEKK3 has been shown to be involved in the regulation of IKK/NF-κB activation (27, 29, 30). MEKK3 is required for LPA-induced NF-κB activation (22). In this study, mutation of Thr-516 and Ser-520 to alanine abolished MEKK3-mediated IKK/NF-κB activation. Therefore, we hypothesized that phosphorylation of Thr-516 and Ser-520 on MEKK3 plays an important role in LPA-mediated IKK/NF-κB activation. To test this hypothesis, HA-MEKK3-WT and HA-MEKK3-AA mutant expression vectors, as well as an empty vector, were stably introduced back into Mekk3−/− MEF cells by the retroviral infection system. Stable MEF cell lines expressing MEKK3-WT (HA-MEKK3-WT), T516A/S520A (HA-MEKK3-AA) mutants, and empty control vector were established. MEKK3 expression was verified by immunoblotting (Fig. 4A).

To further characterize the physiological role of the phosphorylation of Thr-516 and Ser-520 on MEKK3 in LPA- and PKC-mediated IKK/NF-κB activation, Mekk3−/− MEF cell lines reconstituted with control vector, HA-MEKK3-WT, or HA-MEKK3-AA were treated with LPA or PMA/Iono (PKC agonists) at different time points and subsequently lysed. The cell lysates were immunoblotted with the indicated antibodies to examine LPA- and PKC-induced IKK and IκBα phosphorylation. As shown in Fig. 4, A and B, LPA and PMA/Iono induced the phosphorylation of IKKβ and IκBα at a much higher level in the MEKK3-WT-reconstituted MEF cells compared with the MEKK3-AA mutant- and empty vector-reconstituted cells. In addition, LPA and PMA/Iono induced a much higher level of NF-κB nuclear translocation and activation in the MEKK3-WT-reconstituted cells compared with the MEKK3-AA-reconstituted cells (Fig. 4, C–F). These results demonstrate that phosphorylation of Thr-516 and Ser-520 on MEKK3 is required for LPA- and PKC-induced IKKβ phosphorylation, IκBα phosphorylation, and NF-κB activation.

Phosphorylation of Thr-516 and Ser-520 on MEKK3 Is Required for LPA- and PMA/Iono-induced IL-6 Production and Cell Proliferation—MEKK3 has been shown to be required for LPA- and PKC-mediated NF-κB-induced IL-6 production in MEF cells (17, 19, 20). To determine the role of phosphorylation of Thr-516 and Ser-520 on MEKK3 in LPA-induced IL-6 production, the vector control- and HA-MEKK3-WT- or HA-MEKK3-AA-reconstituted Mekk3−/− MEF cell lines were treated with or without LPA or PMA/Iono and then analyzed for IL-6 production in the cells by ELISA (Fig. 5, A and B). In this assay, both LPA and PMA/Iono induced a much higher level of IL-6 production in the MEKK3-WT-reconstituted MEF cells compared with the control and MEKK3-AA-reconstituted cells (Fig. 5, A and B). In addition, we found that LPA- and PMA/Iono-induced cell proliferations were impaired in the vector control- and MEKK3-AA-reconstituted cells compared with the MEKK3-WT-reconstituted MEF cells (Fig. 5, C and D). These results demonstrate that phosphorylation of Thr-516 and Ser-520 on MEKK3 is essential for the LPA- and PKC-induced physiological effect in MEF cells.
DISCUSSION

Our previous study demonstrates that MEKK3 is required for the LPA-induced IKKβ/NF-κB signal transduction pathway (22). However, the molecular regulation of MEKK3-mediated NF-κB activation in LPA signaling remains to be better defined. Here we have presented evidence that both Thr-516 and Ser-520 within the MEKK3 kinase activation loop are two essential regulatory phosphorylation sites for MEKK3-mediated IKKβ/NF-κB activation. This phosphorylation of MEKK3 is confirmed by antibodies that recognize pThr-516 and pSer-520 within the activation loop. Further evidence to support the importance of a negative charge at Thr-516 and Ser-520 on MEKK3 comes from experiments using the T516E and S520D mutants of MEKK3, which act as constitutively active mutants to induce IKKβ/NF-κB activation. We propose that phosphorylation of these two residues within the kinase activation loop is a general mechanism for MEKK3-mediated NF-κB activation.

Previously, PKCδ and PKCγ have been suggested to be involved in LPA-induced NF-κB activation (31). Further studies are needed to determine the mechanism of PKC-mediated MEKK3 activation in LPA signaling.

In conclusion, our data provide evidence that phosphorylation of MEKK3 at Thr-516 and Ser-520 is required for LPA- and PKC-induced IKKβ/NF-κB activation in MEF cells. In view of the data presented here and in previous reports, we propose a working model (Fig. 6) in which phosphorylation of MEKK3 at Thr-516, Ser-520, and Ser-526 induced by LPA-mediated PKC activation is essential for MEKK3-mediated IKKβ/NF-κB activation in the cells.

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