MEKK3 is a central intermediate signaling component in lysophosphatidic acid (LPA)-induced activation of the nuclear factor-κB (NF-κB). However, the precise mechanism for the termination of MEKK3 kinase activity is not fully understood. Using a functional genomic approach, we have identified a protein serine/threonine phosphatase, protein phosphatase 2A (PP2Ac), as a MEKK3 phosphatase. Overexpression of PP2A catalytic subunit (PP2Ac) α-isof orm results in dephosphorylation of MEKK3 at Thr-516 and Ser-520 and termination of MEKK3-mediated NF-κB activation. PP2Ac associates with the phosphorylated form of MEKK3 and the interaction between PP2Ac and MEKK3 is induced by LPA in a transient fashion in the cells. Furthermore, knockdown of PP2Ac expression enhances LPA-induced MEKK3-mediated IκB kinase β (IKKβ) phosphorylation and NF-κB activation. These data suggest that PP2A plays an important role in the termination of LPA-mediated NF-κB activation through dephosphorylating and inactivating MEKK3.

Nuclear factor-κB (NF-κB) is a group of transcription factors that critically regulates cellular responses in immunity, stress responses, anti-apoptosis, cell proliferation, and differentiation (1–4). NF-κB is sequestered in the cytoplasm by members of the inhibitors of NF-κB (IκB) family acting as inhibitory proteins in the unstimulated cells. Stimulation of cells with a variety of extracellular stimuli leads to activation of IκB kinase (IKK) and phosphorylation of IκB proteins by the activated IKK (5–12). Phosphorylation of IκB protein induces its ubiquitination and degradation (1, 13, 14). Degradation of the IκB proteins allows translocation of NF-κB to the nucleus, where it regulates the target gene expression and cellular responses (15).

Lysophosphatidic acid (LPA), a naturally occurring, water-soluble glycerophospholipid, is involved in the regulation of various cellular responses such as cell proliferation, chemotaxis, and survival through binding to its cognate G protein-coupled receptors (GPCRs) and activating LPA receptor-mediated intracellular signaling pathways, including IKKβ/NF-κB (16). Recently, several adaptor and scaffold proteins have been identified as essential intermediate signaling components in LPA-induced NF-κB activation 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) (17–21). Recently, MEKK3 (mitogen-activated protein kinase kinase kinase 3) but not TAK1 (TGF-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).

Phosphorylation of the specific serine or threonine residues within the activation loop of the protein kinase located between kinase subdomains VII and VIII is one of the critical steps for kinase activation (23). In the case of MEKK3, previous studies have demonstrated that phosphorylation of several serine/threonine residues on MEKK3 can be induced by distinct extracellular stimuli. For example, tumor necrosis factor α (TNFα) induces MEKK3 phosphorylation at Ser-166 and Ser-337 residues.
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dues (24) while interleukin-1 (IL-1) induces MEKK3 phosphorylation at Ser-526 residue that is required for MEKK3-mediated mitogen-activated protein (MAP) kinase and NF-κB activation (25, 26). Recently, we found that phosphorylation of Thr-516 and Ser-520 residues in the kinase activation loop of MEKK3 is required for LPA-mediated IKKβ/NF-κB activation (27).

Although a better understanding has been achieved regarding the mechanism of the MEKK3 activation, it remains unclear how MEKK3 activation is down-regulated in the cells and which member(s) of the protein serine/threonine phosphatase family dephosphorylate(s) the conserved residues Thr-516 and Ser-520 within the kinase activation loop of MEKK3 and inhibit(s) MEKK3-mediated signal transduction pathway.

In the human genome, protein serine/threonine phosphatases are mainly composed of two structurally distinct families: PPP and PPM (28, 29). The PPP family, including PPP1, PPP2/PPP2A, PPP3/PPP2B, PPP4, PPP5, PPP6, and PPP7, consists of a highly conserved catalytic domain and distinct regulatory domains or subunits. The PPP family is a group of monomeric metal-ion-dependent phosphatases including PPP1A, PPP1B, PPP1C, PPP1D/wip1, PPP1E, PPP1F, PPP1G, PPP1H, PPP1J, PPP1L, PPP1M, PPP1N, PPP1P, PPP1Q, PPP1R, PPP1S, PPP1T, PPP1U, PPP1V, PPP1W, PPP1X, PPP1Y, PPP1Z, PPP2A, PPP2B, PPP2C, PPP2D, PPP2E, PPP2F, PPP2G, PPP2H, PPP2I, PPP2J, PPP2K, PPP2L, PPP2M, PPP2N, PPP2O, PPP2P, PPP2Q, PPP2R, PPP2S, PPP2T, PPP2U, PPP2V, PPP2W, PPP2X, PPP2Y, PPP2Z, PPP3A, PPP3B, PPP3C, PPP3D, PPP3E, PPP3F, PPP3G, PPP3H, PPP3I, PPP3J, PPP3K, PPP3L, PPP3M, PPP3N, PPP3O, PPP3P, PPP3Q, PPP3R, PPP3S, PPP3T, PPP3U, PPP3V, PPP3W, PPP3X, PPP3Y, PPP3Z, PPP4A, PPP4B, PPP4C, PPP4D, PPP4E, PPP4F, PPP4G, PPP4H, PPP4I, PPP4J, PPP4K, PPP4L, PPP4M, PPP4N, PPP4O, PPP4P, PPP4Q, PPP4R, PPP4S, PPP4T, PPP4U, PPP4V, PPP4W, PPP4X, PPP4Y, PPP4Z, PPP5A, PPP5B, PPP5C, PPP5D, PPP5E, PPP5F, PPP5G, PPP5H, PPP5I, PPP5J, PPP5K, PPP5L, PPP5M, PPP5N, PPP5O, PPP5P, PPP5Q, PPP5R, PPP5S, PPP5T, PPP5U, PPP5V, PPP5W, PPP5X, PPP5Y, PPP5Z, PPP6A, PPP6B, PPP6C, PPP6D, PPP6E, PPP6F, PPP6G, PPP6H, PPP6I, PPP6J, PPP6K, PPP6L, PPP6M, PPP6N, PPP6O, PPP6P, PPP6Q, PPP6R, PPP6S, PPP6T, PPP6U, PPP6V, PPP6W, PPP6X, PPP6Y, PPP6Z, PPP7A, PPP7B, PPP7C, PPP7D, PPP7E, PPP7F, PPP7G, PPP7H, PPP7I, PPP7J, PPP7K, PPP7L, PPP7M, PPP7N, PPP7O, PPP7P, PPP7Q, PPP7R, PPP7S, PPP7T, PPP7U, PPP7V, PPP7W, PPP7X, PPP7Y, PPP7Z. The heterotrimeric protein phosphatase 2A (PP2A), is a ubiquitous and conserved serine/threonine phosphatase with broad substrate specificity and diverse cellular functions. PP2A consists of a dimeric core enzyme comprising of the structural A and catalytic C subunits, and a regulatory B subunit.

In this report, we used a functional genomic approach to identify the MEKK3 phosphatase(s) by screening a library of serine/threonine phosphatases whose overexpression inhibits MEKK3-mediated NF-κB activation and dephosphorylates MEKK3 at the conserved Thr-516 and Ser-520 residues within the kinase activation loop. Here we present evidence that PP2A functions as the MEKK3 phosphatase and terminates LPA and PKC-induced IKKβ/NF-κB activation through dephosphorylating and inactivating MEKK3.

**EXPERIMENTAL PROCEDURES**

**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 recommendation. HeLa cells were cultured in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum, sodium pyruvate (1 mm), and transfected with FuGene HD following the manufacturer’s protocol. The above medium also contained penicillin (100 units/ml), streptomycin (100 mg/ml), and glutamine (2 mm).

**Construction of Human Serine/Threonine Phosphatase Expression Library**—Human serine/threonine phosphatase expression library was made as described previously (30). In brief, human serine/threonine phosphatase clones were purchased from ATCC (Manassas, VA) and Open Biosystems (Huntsville, AL). Full-length cDNA sequence for each phosphatase containing an open reading frame was subcloned into pcDNA3.1 expression vector (Invitrogen). Mammalian PPP1CC expression vector was kindly provided by Dr. Sergei Nekhai (Howard University, Washington, D.C.). PPP1D expression vector was obtained from Dr. Larry Donehower (Baylor College of Medicine) and PPP5C from Dr. Xiaofan Wang (Duke University).

**Expression Plasmids and Small Hairpin RNA (shRNA) Expression Constructs**—The full-length open reading frame of human PP2Ac wild type (WT) was subcloned in-frame into mammalian expression vector pcDNA3.1 with an N-terminalFLAG or Myc tag (Invitrogen). The PP2Ac-D85N mutant expression construct was generated by site-directed PCR mutagenesis (Stratagene) and verified by DNA sequencing. A cDNA construct containing the full-length open reading frame of the wild-type human MEKK3 was subcloned into the HA-tagged mammalian expression vectors pcDNA3.1. HA-MEKK3-T516E/S520D and HA-MEKK3-T516A/S520A mutant expression constructs were generated by site-directed PCR mutagenesis and verified by DNA sequencing as described before (27). Mammalian expression vector for HA-IKKβ was obtained from Dr. Paul Chiao (The University of Texas MD Anderson Cancer Center). The NF-κB-dependent firefly luciferase reporter plasmid and pCMV promoter-dependent Renilla luciferase reporter plasmid were purchased from Clontech (Mountain View, CA). A pSuper-retro vector (Oligoengine) was used to generate shRNA plasmids for PP2Ac. For PP2Ac β-isomorph shRNA, the following target sequences have been selected: 5'-AATTACGTGTTACTGTGTTGGG-3' (sh-PP2Ac-1), 5'-AAGAGGTTCCGTGTCCTCTGTTA-3' (sh-PP2Ac-2). pSuper-shRNA-control is: 5'-CTGCGATCCTGTTGGATGA-3'. The authenticity of these plasmids was confirmed by sequencing.

**Antibodies and Reagents**—Antibodies against HA epitope (F-7) and Myc epitope (9E10) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz Biotechnology); antibodies against Flag epitope, β-actin were from Sigma-Aldrich. Antibodies against phospho-IKKα/β, IKKβ, and secondary antibodies conjugated to horseradish peroxidase were from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies against PP2A, C subunit were from Upstate (Millipore) Company (Billerica, MA). Antibodies against MEKK3 were from BD Biosciences Pharmingen (San Diego, CA). Polyclonal antibodies specific for phosho-human MEKK3 (pThr-516/pSer-520) were produced by immunizing rabbits with MEKK3 phosphopeptide (GASKRLQpTICmpSGTGMR) at Genemed Synthesis, Inc. (San Antonio, TX). LPA, phorbol-12-myristate-13-acetate (PMA), ionomycin (Iono), and polybrene were purchased from Sigma-Aldrich Co. FuGene 6 and FuGene HD transfection reagents were from Roche (Alameda, CA). Lambda Protein Phosphatase (λ-PPase) was purchased from New England Biolabs (Ipswich, MA). Cell culture media were obtained from Invitrogen (Carlsbad, CA). Polyvinylidene difluoride membrane was obtained from Bio-Rad. The ECL-Plus Western blotting system was purchased from GE Healthcare Biosciences Corp. (Piscataway, NJ).

**Luciferase Reporter Gene Assay**—The luciferase reporter gene assay was performed using a dual luciferase reporter assay system (Promega, Madison, WI) and a Monolight 3010 luminometer (BD Biosciences Pharmingen) as described previously (31). Briefly, targeted cells were transiently co-transfected with specific vectors and an NF-κB-dependent firefly luciferase.
Generation of Stable HeLa Cells Expressing shRNA Targeting PP2Ac—The pSuper-sh-PP2Ac retroviral construct was transfected into HEK-293T cells with retrovirus packing vector Peg-pam 3e and RDF vector using FuGene 6 transfection reagent. Viral supernatants were collected after 48 and 72 h. HeLa cells were incubated with virus-containing medium in the presence of 4 μg/ml polybrene (Sigma Aldrich). Stable cell lines were established after 5 days of puromycin (2 μg/ml) selection and knockdown of the target gene was confirmed by Western blotting.

Immunoblotting and Immunoprecipitation—Cells were harvested in ice-cold PBS (pH 7.4) and spun down. The pellets were dissolved in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% IGEPA, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM benzamidine, 20 mM disodium p-nitrophenylphosphate (pNPP), 0.1 mM sodium orthovanadate (OV), 10 mM sodium fluoride, phosphatase inhibitor mixture A and B (Sigma Aldrich Co.)). The cell lysates were either subjected directly to 10% SDS-PAGE for immunoblotting analysis or immunoprecipitated for 3 h with the indicated antibodies. Immune complexes were recovered with protein A-agarose (Santa Cruz Biotechnology) for 3 h, then washed five times with wash buffer containing 20 mM HEPES (pH 7.4), 50 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, and 0.05% Triton X-100. For immunoblotting, the immunoprecipitates or 10% whole cell lysates (WCL) were resolved on SDS-PAGE and transferred to polyvinylidene difluoride membranes. 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 instruction.

In Vitro Phosphatase Assays—HEK-293T cells seeded onto 10-cm dishes were transfected with the HA-MEKK3, Flag-PP2Ac wild type, or Flag-PP2Ac phosphatase-deficient D85N mutant expression plasmid, respectively. The Flag-PP2Ac-WT/D85N proteins were immunoprecipitated from cell extracts with anti-Flag antibodies. The phosphorylated HA-MEKK3 in the cell extracts was incubated with the immunoprecipitated Flag-PP2Ac wild-type or phosphatase-deficient D85N mutant protein in phosphate 2A buffer (25 mM imidazole, pH 7.2, 0.1 mM EGTA, 20 mM MnCl2, 0.01% 2-mercaptoethanol, 1% bovine serum albumin) or λ-Phosphatase at 30 °C for 30 min. The phosphatase reactions were then terminated by boiling in protein sample buffer, and proteins were separated by 10% SDS-PAGE. The levels of HA-MEKK3 phosphorylation were measured by immunoblotting analysis with antibodies against phospho-MEKK3 (pThr-516/pSer-520).

RESULTS

PP2A Is a MEKK3 Phosphatase—Our previous studies demonstrate that MEKK3 phosphorylation at Thr-516 and Ser-520 residues within the kinase activation loop is essential for MEKK3-mediated IKK/NF-κB activation (27). However, the identity of phosphatase(s) that control(s) MEKK3 activity through Thr-516/Ser-520 residues remains to be determined.

To identify which phosphatase(s) are involved in dephosphorylating and down-regulating MEKK3-mediated NF-κB activation, we co-transfected HA-tagged MEKK3 expression plasmid into HEK-293T cells with expression vectors encoding twenty two human protein serine/threonine phosphatases (catalytic subunits if it is multimeric) including 10 PPPs and 12 PPMs. Then we used an NF-κB luciferase reporter assay to assess the effect of overexpression of each phosphatase on MEKK3-induced NF-κB activation. In this screen, as shown in Fig. 1A, PP2Ac as well as PPM1A and PPM1B almost completely abolished MEKK3-induced NF-κB activation whereas other phosphatases had either no or less effect. To validate our results from the above screening assay, we chose the phosphatases with some degree of inhibition on MEKK3-induced NF-κB activation and examined the effects of overexpression of these phosphatases including PP2Ac, PPM1A, and PPM1B on the phosphorylation status of Thr-516 and Ser-520 at the MEKK3 kinase activation loop (Fig. 1B). Immunoblotting analysis of cell lysates revealed that the phosphorylation level of HA-MEKK3 at Thr-516 and Ser-520 was markedly reduced in the cells co-transfected with PP2Ac compared with the cells co-transfected with vector alone whereas total MEKK3 levels were similar in control cells and the cells with overexpression of other phosphatases (Fig. 1B). These results suggest that PP2A specifically dephosphorylates MEKK3 at Thr-516 and Ser-520 residues in cells. To assess whether the effects of PP2Ac on MEKK3 are due to their phosphatase activity, we generated expression vectors encoding PP2Ac phosphatase-deficient Asp-85 to Asn (D85N) mutant and found that only wild-type PP2Ac, but not phosphatase-deficient D85N mutant, abolished the phosphorylation of Thr-516 and Ser-520 at the activation loop of MEKK3 (Fig. 1C) and the MEKK3-mediated NF-κB activation (Fig. 1D). Consistent with above results, we found that overexpression of PP2Ac was able to abolish the wild-type MEKK3-induced NF-κB activation almost completely whereas it failed to terminate the NF-κB activation induced by the MEKK3 T516E/S520D constitutively active mutant (Fig. 1E). Consistent with our earlier report that PPM1A and PPM1B act as IKKβ phosphatases (30), we found that overexpression of PP2Ac had no effect on the phosphorylation of IKKβ at its conserved residues Ser-177/Ser-181 (Fig. 1F), suggesting that PP2A specifically targets on MEKK3 but not IKKβ to inhibit MEKK3-mediated NF-κB activation.

To further determine whether PP2A is able to dephosphorylate the activated MEKK3 directly, we examined the phosphorylation status of the phosphorylated MEKK3 incubated with immunoprecipitated Flag-PP2Ac in vitro. In this assay, HA- MEKK3 was overexpressed in HEK-293T cells, and phosphorylated HA-MEKK3 in the cell extracts was incubated with the
FIGURE 1. PP2A is a MEKK3 phosphatase. A, effect of overexpression of members of serine/threonine phosphatases or the catalytic subunit of polymeric phosphatases on the MEKK3-induced NF-κB activation. HA-MEKK3 expression plasmid, NF-κB luciferase reporter plasmid and Renilla luciferase plasmid were co-transfected into HEK-293T cells with empty vector or different phosphatase expression plasmids. The relative luciferase activity was measured 36 h later and normalized with the Renilla activity. Error bars indicate ± S.D. in triplicate experiments. B, effect of overexpression of members of serine/threonine phosphatases or the catalytic subunit of polymeric phosphatases on the MEKK3 phosphorylation at Thr-516 and Ser-520. Cell extracts were prepared from HEK-293T cells co-transfected with expression vectors for HA-MEKK3 and different expression vectors for phosphatases as indicated and analyzed by immunoblotting with anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies and re-probed with anti-MEKK3 antibodies to detect the expression level of HA-MEKK3. Then the WCL were immunoblotted with anti-Flag antibodies to detect the presence of PP2A-WT or -D85N mutant protein expression. D, PP2A phosphatase activity is required for its effect on MEKK3-induced NF-κB activation. HA-MEKK3 expression plasmid, NF-κB luciferase reporter plasmid and Renilla luciferase plasmid were co-transfected into HEK-293T cells with empty vector or expression vectors for both PP2A-WT and -D85N mutant. The relative luciferase activity was measured 36 h later and normalized with the Renilla activity. Error bars indicate ± S.D. in triplicate experiments. E, MEKK3 constitutively active mutant is resistant to the down-regulation of MEKK3-mediated NF-κB activation by PP2Ac. Expression plasmid encoding HA-MEKK3 wild type or constitutively active mutant (T516E/S520D) was transfected into HEK-293T cells co-transfected with PP2 Ac-WT or -D85N mutant and NF-κB luciferase reporter and Renilla luciferase plasmid. The relative luciferase activity was measured 36 h later and normalized with the Renilla activity. Error bars indicate ± S.D. in triplicate experiments. F, PP2A does not dephosphorylate IKKβ at Ser-177 and Ser-181 residues. Cell extracts were prepared from HEK-293T cells co-transfected with expression vectors for the HA-tagged IKKβ and Flag-tagged PP2Ac-WT or -D85N mutant, and immunoprecipitated with anti-HA antibodies, then analyzed by immunoblotting with anti-phospho-IKKβ (pSer-177/pSer-181) antibodies, after that the blot was stripped and re-probed with anti-HA antibodies to confirm that equivalent levels of HA-tagged IKKβ. WCL were immunoblotted with anti-HA and anti-Flag antibodies to detect the expression levels of HA-IKKβ and Flag-PP2A-WT or -D85N mutant in the cells.

G, PP2A dephosphorylates MEKK3 in vitro. HA-MEKK3 expression vector was transfected into HEK-293T cells, and the phosphorylated MEKK3 were incubated with immunoprecipitated Flag-PP2Ac-WT or -D85N mutant protein, or λ-PPase for 30 min before being analyzed by immunoblotting with the anti-phospho-MEKK3 (pThr-516/pSer-520) antibodies and re-probed with anti-MEKK3 antibodies to detect the protein level of HA-MEKK3. The PP2Ac-WT and -D85N proteins used in above assays were detected with anti-Flag antibodies.
immunoprecipitated Flag-PP2Ac or its phosphatase-deficient D85N mutant protein, or λ-PPase as a control. The phosphorylation level of HA-MEKK3 was found to be significantly decreased by co-incubation with immunoprecipitated Flag-PP2Ac wild type, as well as with λ-PPase, but not phosphatase-deficient D85N mutant protein (Fig. 1G). These results demonstrate that PP2A targets on the phosphorylated Thr-516 and Ser-520 within the activation loop of MEKK3. Taken together, our results strongly suggest that PP2A acts as a MEKK3 phosphatase to terminate MEKK3-mediated IKKβ/NF-κB activation.

PP2Ac Binds to the Phosphorylated MEKK3—To assess whether MEKK3 interacts with PP2A, the expression vectors encoding HA-tagged MEKK3 wild-type or T516/S520D mutant were co-transfected with vectors encoding Myc-tagged PP2Ac wild-type or -D85N mutant into HEK-293T cells. Then Myc-tagged PP2Ac wild type or -D85N mutant protein was immunoprecipitated from cell lysates with anti-Myc antibodies and immunoblotted with anti-HA antibodies. Interestingly, PP2Ac-D85N mutant pulled down more HA-MEKK3 compared with PP2Ac wild type (Fig. 2A). Furthermore, PP2Ac-D85N mutant pulled down much more HA-MEKK3-T516/S520D mutant compared with PP2Ac wild type (Fig. 2A). Consistent with the above results, we found that the MEKK3 with double alanine mutations at Thr-516 and Ser-520 (MEKK3-T516A/S520A) residues could not bind either wild-type or -D85N mutant PP2Ac (data not shown). These results suggest that only phosphorylated MEKK3 binds to PP2A as its substrate.

Previous studies suggest that LPA and PKC agonists induce MEKK3 phosphorylation and activation (22, 27). We further examined whether LPA and PKC agonists induce the association of MEKK3 with PP2A. In this assay, HEK-293T cells co-transfected with HA-MEKK3 and Myc-PP2Ac expression plasmids were treated with LPA for the time periods as indicated (Fig. 2B). The Myc-tagged PP2Ac in the cell lysates were immunoprecipitated with the antibodies against Myc epitope and immunoblotted with anti-HA antibodies to detect the presence of HA-tagged MEKK3. As shown in Fig. 2B, LPA rapidly induced association of MEKK3 wide type but not -T516A/S520A mutant with PP2Ac. These results suggest that LPA-induced MEKK3 phosphorylation and activation results in the binding of PP2Ac to the phosphorylated MEKK3. Consistent with this result, we also found that PKC agonists (PMA/Iono) also induced interaction of MEKK3 wide type with PP2Ac (Fig. 2C).

Suppression of PP2Ac Expression Enhances LPA-induced IKK/NF-κB Activation—LPA and PKC agonists-induced IKKβ/NF-κB activation requires MEKK3 phosphorylation at Thr-516 and Ser-520 residues (27). To further address the role of PP2A in LPA-induced IKKβ phosphorylation and NF-κB activation, we generated shRNA expression vectors for knocking down the expression of PP2Ac and found that PP2Ac expression can be suppressed by these shRNA expression vectors (Fig. 3A). And that two sh-PP2Ac expression plasmids effectively rescued the inhibition of MEKK3-mediated NF-κB reporter activity by overexpression of PP2Ac (Fig. 3B). Subsequently we found that co-transfection of HA-MEKK3 with sh-PP2Ac expression vectors in HeLa cells resulted in a higher MEKK3-induced NF-κB activity in an NF-κB-dependent luciferase reporter assay (Fig. 3C). We then generated PP2Ac stable knockdown HeLa cell lines using a retroviral transduction system and analyzed the effect of PP2Ac knockdown on the LPA-induced IKKβ phosphorylation. In this assay, HeLa cells with sh-control and sh-PP2Ac stable expression were treated with LPA or PMA/Iono (PKC agonists) for the different time periods as indicated and subsequently lysed (Fig. 3, D and E). We found that knockdown of PP2Ac expression caused the enhanced phosphorylation of IKKβ at the early time points of LPA and PMA/Iono stimulation. Consistent with the above results, knockdown of PP2Ac expression in HeLa cells resulted in a higher LPA or PKC-induced NF-κB activation in an NF-κB-dependent luciferase reporter assay (Fig. 3, F and G).
together, these results demonstrate that PP2A is responsible for terminating LPA-induced MEKK3 phosphorylation and activation as well as inhibition of MEKK3-mediated IKK\(\beta\)/NF-\(\kappa\)B activation in the cells.

We further examined the role of PP2A in IKK\(\beta\)/NF-\(\kappa\)B activation mediated by other signaling pathways. In these assays, we found that suppression of PP2Ac expression by shRNA in HeLa cells enhanced TNF\(\alpha\)-, IL-1\(\beta\)-, and LPS-induced IKK
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FIGURE 4. A working model for MEKK3 dephosphorylation and inactivation mediated by PP2A. LPA induces MEKK3 phosphorylation and activation as well as MEKK3-mediated IKKβ/NF-κB activation. PP2Ac binds to the phosphorylated MEKK3 and subsequently dephosphorylates MEKK3 at Thr-516, Ser-520, and Ser-526 residues to terminate MEKK3-mediated IKKβ/NF-κB activation.

Phosphorylation and IL-6 mRNA expression (supplemental Fig. S1, A and D). In Jurkat cells, suppression of PP2Ac expression also enhanced PKC agonist (P/I)-induced IKK phosphorylation and IL-6 mRNA expression compared with the control cell line (supplemental Fig. S1, B, C, and E). These results suggest that PP2A plays an important role in the negative regulation of IKKβ/NF-κB activation mediated by diverse signaling pathways.

DISCUSSION

Phosphorylation of MEKK3 at Thr-516 and Ser-520 within the kinase activation loop is required for LPA-induced IKKβ/NF-κB activation. Following LPA stimulation, MEKK3 phosphorylation and activation is rapidly induced and in turn leads to IKK/NF-κB activation (22, 27). However, the mechanism of MEKK3 dephosphorylation and inactivation following LPA stimulation to attenuate LPA-induced NF-κB activation remains to be defined clearly.

It is not clear which phosphatase(s) are truly MEKK3 phosphatase(s) that down-regulate(s) MEKK3 kinase activity through the dephosphorylation of MEKK3 at the conserved residues Thr-516 and Ser-520 within the kinase activation loop. Therefore, we decided to take a functional genomic approach to further analyze the mechanism of MEKK3 inactivation and identify MEKK3-specific phosphatase(s). In this study, we identify that PP2Ac is a major phosphatase involved in negatively regulating MEKK3 phosphorylation and activation. We demonstrate that PP2Ac is essential to terminate MEKK3-mediated IKK/NF-κB activation through binding to the activated form of MEKK3 and dephosphorylating MEKK3 at Thr-516 and Ser-520. Our studies suggest that PP2Ac functions as a MEKK3 phosphatase and serves as an important Yin-Yang regulatory mechanism to maintain a delicate balance in LPA-mediated cellular responses.

In this investigation, we found that only PP2Ac phosphatase-deficient mutant but not the wild type were able to pull-down the phosphorylated MEKK3, as demonstrated by our co-transfection and immunoprecipitation assays. These data suggest that PP2Ac only binds to the phosphorylated MEKK3. In addition, LPA induces the interaction between MEKK3 and PP2A in a temporary fashion. However, we could not show the LPA-induced endogenous MEKK3 and PP2Ac binding possibly due to lack of good antibodies for immunoprecipitation of MEKK3 and PP2Ac. Together, these results suggest that LPA-induced MEKK3 activation is rapidly terminated by PP2A through physical binding to and dephosphorylating MEKK3.

Several kinases have been identified to be the substrates for PP2A including calcium-calmodulin-dependent protein kinase IV (CaMKIV) (32), p70 S6 kinase (33, 34), p21-activated kinases (PAK) (33), p38 kinase (35), casein kinase II (CK2) (36), kinase suppressor of Ras1 (KSR1) (37), Raf1-mitogen-activated protein kinase (MAPK) (37, 38), and protein kinases B (PKB)/AKT (39–43), nuclear ribosomal protein S3 (rpS3) (44), spingosine kinase 1 (SK1) (45), p107 (46), Pim-1 (47), and polo-like kinase 1 (PLK-1) (48).

In our study, we found that suppression of PP2Ac expression in HeLa cells enhanced both LPA- and P/I-induced ERK and JNK phosphorylation (supplemental Fig. S2). However, suppression of PP2Ac expression in HeLa cells did not affect both LPA- and P/I-induced AKT1 phosphorylation at Thr-308 (supplemental Fig. S2). It is likely that LPA and P/I-induced AKT1 phosphorylation and activation is not negatively regulated by PP2Ac.

Interestingly, PP2A has been suggested to be able to dephosphorylate MEKK3 at the Ser-526 residue (26). Consistent with this report, we found that PP2A also dephosphorylated MEKK3 at Thr-516 and Ser-520 residues. Together, PP2A is able to dephosphorylate MEKK3 at the above three sites to inactivate MEKK3-mediated downstream signal transduction.

In conclusion, our data provide evidence of the physical and functional interaction between MEKK3 and PP2A. In view of the data presented here and in previous reports, we propose a working model (Fig. 4), in which upon LPA-induced MEKK3 phosphorylation at Thr-516, Ser-520, and Ser-526, PP2A would bind to the phosphorylated MEKK3 and act as a MEKK3 phosphatase to inactivate LPA-induced MEKK3 activation as well as MEKK3-mediated IKKβ/NF-κB activation. This report demonstrates that PP2A terminates LPA-induced IKKβ/NF-κB activation through dephosphorylating and inactivating MEKK3.

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