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PPM1A and PPM1B act as IKKβ phosphatases to terminate TNFα-induced IKKβ-NF-κB activation

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Abstract

IKKβ serves as a central intermediate signaling molecule in the activation of the NF-κB pathway. However, the precise mechanism for the termination of IKKβ activity is still not fully understood. Using a functional genomic approach, we have identified two protein serine/threonine phosphatases, PPM1A and PPM1B, as IKKβ phosphatases. Overexpression of PPM1A or PPM1B results in dephosphorylation of IKKβ at Ser177 and Ser181 and termination of IKKβ-induced NF-κB activation. PPM1A and PPM1B associate with the phosphorylated form of IKKβ, and the interaction between PPM1A/PPM1B and IKKβ is induced by TNFα in a transient fashion in the cells. Furthermore, knockdown of PPM1A and PPM1B expression enhances TNFα-induced IKKβ phosphorylation, NF-κB nuclear translocation and NF-κB-dependent gene expression. These data suggest that PPM1A and PPM1B play an important role in the termination of TNFα-mediated NF-κB activation through dephosphorylating and inactivating IKKβ.

Keywords

Tumor necrosis factor; Kinase; Phosphatase; IκB kinase; NF-κB; Signal transduction

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1. Introduction

The NF-κB family of transcription factors plays critical roles in controlling inflammation, immune response, and anti-apoptosic responses [1–3]. Stimulation of various cell surface receptors, including receptors for proinflammatory cytokines such as tumor necrosis factor (TNFα) and Interleukin-1 (IL-1β), Toll-like receptors (TLRs), antigen receptors, and G protein-coupled receptors (GPCRs), activates NF-κB [4]. In unstimulated cells, NF-κB is sequestered in the cytoplasm by its inhibitory proteins, which are members of the IκB family. Cell stimulation results in phosphorylation of IκB proteins and subsequently rapid ubiquitination and degradation through the 26S proteasome [5–7]. Degradation of the IκB proteins liberates NF-κB and allows its translocation to the nucleus, where it controls the expression of the target genes [8].

Phosphorylation of IκB protein is achieved by the activated IκB kinase (IKK) [9–16]. The activation of the IKK complex composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO, is the convergence point for many NF-κB signaling pathways and activity of IKKs is regulated by phosphorylation [17,18]. Gene knockout studies indicate that IKKβ is the catalytic subunit required for activation of NF-κB in response to TNFα[19,20].

One of the critical steps for the kinase activation is the phosphorylation of the specific serine or threonine residues within the activation loop of the protein kinase located between kinase subdomains VII and VIII. Activation of IKKβ requires phosphorylation of the conserved residues Ser177 and Ser181 within the kinase activation loop. Mutation of these serine residues to alanine markedly decreases IKKβ activity, whereas replacement of these serine residues with glutamates results in the generation of constitutively active kinases [17,18].

Although significant progress has been made on the mechanism of the IKKβ activation, it is unclear how IKKβ activation is down-regulated in the cells and which member of the protein serine/threonine phosphatase family dephosphorylates the conserved residues Ser177 and Ser181 within the kinase activation loop of IKKβ. Several phosphatases including PPM1B have been shown to be able to regulate IKKβ activity [16,18,21–23]. However, the identity of the protein serine/threonine phosphatases that dephosphorylate IKKβ and inhibit its activity remains to be clearly defined.

Protein serine/threonine phosphatases in the human genome are mainly composed of two structurally distinct families: PPP and PPM/PP2C [24,25]. The PPP family, including PPP1, PPP2/PP2A, PPP3/PP2B, PPP4, PPP5, PPP6 and PPP7, consists of a highly conserved catalytic domain and distinct regulatory domains or subunits. The PPM family is a group of monomeric metal-ion-dependent phosphatases including PPM1A, PPM1B, PPM1C, PPM1D/wip1, PPM1E, PPM1F, PPM1G, PPM1H, PPM1J, PPM1L, PPM1K, PPM1M, PHLPP, PPTC7, PPM2C.

In this report, we used a functional genomic approach to identify the IKKβ phosphatase by screening a library of serine/threonine phosphatases whose overexpression inhibits IKKβ-mediated NF-κB activation and dephosphorylates IKKβ at the conserved residues Ser177 and Ser181 within the kinase activation loop. Here we present evidence that PPM1A/PP2Cα and PPM1B/PP2Cβ function as the IKKβ phosphatases that dephosphorylate IKKβ and terminate IKKβ-mediated NF-κB activation.

2. Materials and methods

2.1. Cell culture and transfection

HEK 293 and HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and transfected with FuGene 6 (Roche) according to the manufacturer's recommendation. HeLa cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS, sodium pyruvate (1 mM) and transfected with FuGene HD (Roche) following the manufacturer's protocol. The above media also contained penicillin (100 U/ml), streptomycin (100 mg/ml) and glutamine (2 mM).

2.2. Construction of Human serine/threonine phosphatase Expression Library

Human serine/threonine phosphatase clones were purchased from ATCC and Open Biosystem. 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 DC), PPM1D was obtained from Dr. Larry Donehower (Baylor College of Medicine, TX), and PPP5C from Dr. Xiaofan Wang (Duke University, NC).

2.3. Expression plasmids and small hairpin RNA expression constructs

The full-length open reading frame of the wildtype human PPM1A and PPM1B were subcloned in frame into mammalian expression vector pcDNA3.1 with an N-terminal 3Myc tag (Invitrogen). The PPM1A (R174G) and PPM1B (R179G) mutant expression constructs were generated by site-directed PCR mutagenesis (Stratagene) and verified by DNA sequencing. Mammalian expression vector for HA-IKKβ was obtained from Dr. Paul Chiao (The University of Texas MD Anderson Cancer Center, TX). The NF-κB-dependent *firefly* luciferase reporter plasmid and pCMV promoter-dependent *Renilla* luciferase reporter plasmid were purchased from Clontech (Mountain View, California). For bacterial expression of both PPM1A and PPM1B proteins, cDNAs encoding the wildtype (GST-PPM1A-wt and GST-PPM1B-wt) and phosphatase-deficient mutant version (GST-PPM1A-R174G and GST-PPM1B-R179G) of these two proteins were subcloned into pGEX-KG vector (Invitrogen) to generate glutathione *S*-transferase (GST) fusion proteins. A pSuper-retro vector (Ambion) was used to generate shRNA plasmids for PPM1A and PPM1B. For PPM1A, the following target sequences have been selected: 5′-AAGAGGAATGTTATTGAAGCC-3′ (shPPM1A-1), 5′-AAGTACCTGGAATGCAGAGTA-3′ (shPPM1A-2); and for PPM1B, target sequences were 5′-AATGCAGGAAAGCCATACTGA-3′ (shPPM1B-1), 5′-AACTTCTGGAGGAGATGCTGA-3′ (shPPM1B-2); pSuper-shRNA-control is: 5′-CTGGCATCGGTGTGGATGA-3′. The authenticity of these plasmids was confirmed by sequencing.

2.4. Antibodies and reagents

Antibodies against HA epitope, Myc epitope, NF-κB-p65, PCNA (PC-10) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-β-actin antibody was from Sigma-Aldrich Co. (St. Louis, MO). Antibodies against Phospho-IKKα/β and IKKβ were from Cell Signaling Technology, Inc. (Danvers, MA). Antibody against PPM1A was from Abcam Inc. (Cambridge, MA), and antibody against PPM1B was from Bethyl Laboratories, Inc. (Montgomery, TX). Recombinant human TNFα was purchased from the R & D Systems (Minneapolis, MN). FuGene 6 and FuGene HD transfection reagents were from Roche (Alameda, CA). Cell culture media were obtained from Invitrogen (Carlsbad, CA). Nitrocellulose membrane was obtained from Bio-Rad (Hercules, CA).

2.5. Luciferase reporter gene assays

The luciferase reporter gene assay was performed using a dual luciferase reporter assay system (Promega, Madison, WI) and a Monolight 3010 luminometer (BD PharMingen, San Diego, CA) as described previously [26]. Briefly, targeted 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 hrs post-transfection and the luciferase activities were determined. Relative NF-κB luciferase activity was normalized to *Renilla* luciferase activity. Data are presented as the mean ± standard deviation and are representative of three independent experiments.

2.6. Quantitative reverse transcription PCR (qRT-PCR) analyse

Total RNAs were prepared using TriZol reagent (Invitrogen) from HeLa pSuper-shRNA-control, pSuper-shPPM1A and pSuper-shPPM1B cells. qRT-PCR was carried out by using 100 ng of total RNA. A volume of 10 μl of 2x QuantiTect SYBR Green RT-PCR Master Mix (Qiagen), 0.2 μl QuantiTect RT Mix (Qiagen), 1 μl of 10 μM forward and reverse primers, and 6.8 μl of RNase-Free Water were added to each sample for analysis by absolute quantification. qRT-PCR was performed in 96-well plates with the DNA Engine Opticon™ System (MJ Research). The mRNA levels of target genes in the samples were normalized against β-actin. Each target gene was measured in triplicate. The primers were designed by using the Primer3.0 software and are as follows: IL-6: 5′-CACACAGACAGCCACTCACC-3′ and 5′-TTTTCTGCCAGTGCCTCTTT-3′; β-actin: 5′-ACCGCGAGAAGATGACCCAG-3′ and 5′-TTAATGTCACGCACGATTTCCC-3′. Human IL-6 expression in different HeLa stable cell lines was also analyzed by RT-PCR. In this assay, and cDNA was prepared from the total RNA isolated with TriZol Reagent, using SuperScript III Gene Expression Tools (Invitrogen) according to the manufacturer's protocol. PCR was performed on 1 μl aliquots from each cDNA reaction, using human IL-6 and β-actin primer sets (IL-6, 30 cycles; β-actin, 20 cycles). The PCR products were subjected to electrophoresis on a 2% agarose gel.

2.7. Generation of stable HeLa cells expressing shRNA targeting PPM1A or PPM1B

The pSuper-PPM1A or PPM1B retroviral construct was transfected into HEK 293T cells with retrovirus packing vector Pegpam 3e and RDF vector using FuGene 6 transfection reagent. Viral supernatants were collected after 48 and 72 hours. HeLa cells were incubated with virus-containing medium in the presence of 4 mg/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.

2.8. Preparation of nuclear and cytosolic fractions

Nuclear and cytosolic extracts were made as described [27]. In brief, cells were harvested in ice-cold PBS (pH 7.4) and were pelleted by 500 ×g for 3 min and then lysed for 30 min on ice in buffer B (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 (PMSF)). Cell lysates were centrifuged at 15,000×g for 15 min, 4°C. The resulting supernatants constituted cytosolic fractions. The pellets were washed three times with buffer B and then resuspended in buffer C (20 mM HEPES buffer, pH 7.9, containing 400 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF) and incubated for 30 min on ice, then centrifuged at 15,000×g for 15 min. The supernatants were used as nuclear extracts.

2.9. 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% IGEPAL, 0.25% Na-deoxycholate, 1 mM PMSF, 1 mM DTT, 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 (NaF), phosphatase inhibitor cocktail A and B (Sigma Aldrich)). The cell lysates were either subjected directly to 10% SDS-PAGE for immunoblotting analysis or immunoprecipitated for 3 hrs with the indicated antibodies. Immune complexes were recovered with protein A/G-agarose (Santa Cruz Biotechnology) for 3 hrs, then washed three times with wash buffer containing 20 mM HEPES (pH 7.4), 50 mM NaCl, 2.5 mM $MgCl_2$, 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 nitrocellulose 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 (GE Healthcare Biosciences Corp., USA) according to the manufacturer's instruction.

2.10. Purification of GST-PPM1A and PPM1B fusion proteins

All the above-mentioned GST plasmids (GST-PPM1A-wt, GST-PPM1A-R174G, GST-PPM1B-wt and GST-PPM1B-R179G) were transformed into *E. coli* BL-21 strain (Invitrogen), and then the bacteria were grown in Luria broth at 37°C to an A600=0.6 before induction with 0.1 mM isopropyl β-d-thiogalactoside (IPTG) for 4 hrs at 30°C. Bacteria were pelleted and lysed with extraction buffer (50 mM Tris–HCl, pH 8.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mg/ml lysozyme, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF) 45 min on ice. The bacteria were sonicated at 4°C in 1% Sarcosyl (Sigma Aldrich), after which Triton X-100 (1%), 5 μg/ml DNase, and 5 μg/ml RNase (Roche) were added. The lysates were centrifuged at 15,000×g and the supernatants containing GST fusion proteins were collected. Fusion proteins were purified from cell lysates using glutathione–sepharose beads (Sigma Aldrich) overnight at 4°C. The beads were washed three times in extraction buffer containing 0.5% Triton X-100, one time in extraction buffer containing 0.1% Triton X-100. Proteins were eluted in elution buffer (30.7% glutathione, 50 mM Tris-HCl, pH 8.0, 20% glycerol, 5M NaCl) and dialyzed in PBS. The protein concentrations were then assessed with a Bradford Protein Assay (Bio-Rad). The proteins were visualized by 10% SDS-PAGE and Coomassie blue staining of the gel.

2.11. Phosphatase Assays

HEK 293T cells seeded onto 10 cm dishes were transfected with the HA-IKKβ expression plasmid. The HA-IKKβ proteins were immunoprecipitated from cell extracts with anti-HA antibody. After washing beads three times with the wash buffer, the immunoprecipitated HA-IKKβ were then incubated with or without recombinant GST-PPM1A or GST-PPM1B wildtype or phosphatase-deficient mutant proteins in phosphatase 2C buffer (250 mM imidazole, pH 7.2, 1 mM EGTA, 25 mM $MgCl_2$, 0.1% 2-mercaptoethanol, 10% BSA) or Lambda Protein Phosphatase (λ-Ppase) 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-IKKβ phosphorylation were measured by immunoblotting analysis with antibody against phospho-IKKβ.

3. Results

3.1. PPM1A and PPM1B are IKKβ Phosphatases

Phosphorylation of Ser177 and Ser181 at the activation loop of IKKβ is essential for activation of IKKβ by TNFα [18]. We hypothesized that the potential IKKβ phosphatase targeting these two sites might be a member of protein serine/threonine phosphatase family. We then generated a library of mammalian expression vectors that encode 23 protein serine/threonine phosphatases (catalytic subunits if it is multimeric) including 11 PPPs and 12 PPMs. In order to validate the expression of our phosphatase library, we also made a library of phosphatases

with Myc tag and found that they all can be overexpressed once transfected in HEK-293T cells (data not shown). However, to avoid the possibility that the fused Myc tag affects the phosphatase function, we used the library of phosphatases without Myc tag in our screening. Then we used an NF-κB luciferase reporter assay to assess the effect of overexpression of each phosphatase on IKKβ-induced NF-κB activation. In this screen, as shown in Fig. 1A, both PPM1A and PPM1B almost completely abolished IKKβ-induced NF-κB activation whereas other phosphatases had either no or less effect. To validate our results from the screening assay, we chose the phosphatases with some degree of inhibition on IKKβ-induced NF-κB activation and examined the effects of overexpression of these phosphatases including PPM1A and PPM1B on the phosphorylation status of Ser177 and Ser181 at the IKKβ kinase activation loop. Consistent with our reporter screening assay, overexpression of both PPM1A and PPM1B abolished the phosphorylation of IKKβ at Ser177 and Ser181 (Fig. 1B). To assess whether the effects of PPM1A and PPM1B on IKKβ are due to their phosphatase activity, we generated expression vectors encoding both PPM1A and PPM1B phosphatase-deficient Arg (R) to Gly (G) mutants and found that only wildtype PPM1A and PPM1B, but not phosphatase-deficient mutants, abolished the phosphorylation of Ser177 and Ser181 at the activation loop of IKKβ (Fig. 1C) and the IKKβ-induced NF-κB activation (Fig. 1D). To further test the role of PPM1A and PPM1B on NF-κB activation induced by wildtype- or S177E/S181E constitutively active mutant-IKKβ. We found that both PPM1A and PPM1B were able to abolish the wildtype IKKβ-induced NF-κB activation almost completely whereas they failed to terminate the NF-κB activation induced by the IKKβ constitutively active mutant (Fig. 1E). PPM1B has actually been reported to be involved in the regulation of TAK1 phosphorylation and activation [27]. To further determine whether PPM1A and PPM1B are able to dephosphorylate the activated IKKβ directly, we examined the phosphorylation status of the phosphorylated IKKβ incubated with purified recombinant GST-PPM1A and GST-PPM1B in vitro. In this assay, HA-IKKβ was overexpressed in HEK 293T cells, and phosphorylated HA-IKKβ was immunoprecipitated from cell extracts with anti-HA antibody and incubated with recombinant GST-PPM1A or GST-PPM1B or their respective phosphatase-deficent mutants (R/G), or λ-PPase as a control. The phosphorylation level of HA-IKKβ was found to be significantly decreased by co-incubation with both GST-PPM1A and GST-PPM1B wildtype proteins, as well as with λ-PPase, but not phosphatase-deficient mutant proteins (Fig. 1F). These results demonstrate that PPM1A and PPM1B target on the phosphorylated Ser177 and Ser181 within the activation loop of IKKβ. Taken together, our results strongly suggest that PPM1A and PPM1B are IKKβ phosphatases and responsible for terminating IKKβ-mediated NF-κB activation.

3.2. PPM1A and PPM1B bind to the phosphorylated IKKβ

To assess whether IKKβ interacts with PPM1A and PPM1B, the expression vectors encoding HA-tagged IKKβ were co-transfected with vectors encoding Myc-tagged PPM1A or PPM1B wildtype or mutant PPM1A or PPM1B into HEK 293T cells. Then Myc-tagged PPM1A or PPM1B wildtype or mutant proteins were immunoprecipitated from cell lysates with anti-Myc antibody and immunoblotted with anti-HA antibody. Interestingly, only the mutant but not the wildtype Myc-tagged PPM1A or PPM1B pulled down HA-tagged IKKβ (Fig. 2A). Consistent with the above results, we found that the IKKβ with serine to alanine double mutation at Ser177 and Ser181 could not bind either wild-type or mutant PPM1A and PPM1B (data not shown). These results suggest that only phosphorylated IKKβ binds to PPM1A and PPM1B as the substrate for these two phosphatases. To further evaluate these bindings, the transfected cells as described above were treated with TNFα for the time periods as indicated (Fig. 2B). The Myc-tagged PPM1A and PPM1B in the cell lysates were immunoprecipitated with the antibody against Myc epitope and immunoblotted with anti-HA antibody to detect the presence of HA-tagged IKKβ. As shown in Fig. 2B, TNFα rapidly induced co-immunoprecipitation of IKKβ and PPM1A or PPM1B within 5 min. These results suggest that TNFα-induced IKKβ

phosphorylation and activation results in the binding of both PPM1A and PPM1B to the phosphorylated IKKβ.

3.3. Suppression of PPM1A and PPM1B expression enhances TNFα-mediated NF-κB activation

TNFα induces a strong NF-κB activation through the phosphorylation and activation of IKKβ [2,18]. To further address the role of PPM1A and PPM1B in TNFα-induced IKKβ phosphorylation and IKKβ-NF-κB activation, we generated short hairpin RNA (shRNA) expression vectors for knocking down the expression of PPM1A and PPM1B and found that PPM1A and PPM1B expression can be suppressed by these shRNA expression vectors (Fig. 3A). Subsequently we found that co-transfection of HA-IKKβ with sh-PPM1A and sh-PPM1B expression vectors in HeLa cells resulted in a higher IKKβ-induced NF-κB activation in an NF-κB-dependent luciferase reporter assay (Fig. 3B). We then generated PPM1A and PPM1B stable knockdown HeLa cell lines using a retroviral transduction system and analyzed the effect of both PPM1A and PPM1B knockdown on the TNFα-induced IKKβ phosphorylation and NF-κB nuclear translocation. In this assay, HeLa cells with sh-control, sh-PPM1A and sh-PPM1B stable expression were then treated with TNFα for the different time periods as indicated and subsequently lysed (Fig. 3C and 3D). We found that knockdown of PPM1A and PPM1B expression caused the enhanced phosphorylation of IKKβ at the early time points of TNFα stimulation and sustained phosphorylation of IKKβ at the later time points of stimulation (Figure 3C). Nuclear extracts from these cells treated at different time points were prepared and immunoblotted with an antibody specific for NF-κB-p65. We found that knockdown of PPM1A and PPM1B expression resulted in sustained NF-κB nuclear localization at the later time points of stimulation (Figure 3D). Consistent with the above results, knockdown of PPM1A and PPM1B expression in HeLa cells resulted in a higher TNFα-induced NF-κB activation in an NF-κB-dependent luciferase reporter assay (Fig. 3E). Taken together, these results demonstrate that PPM1A and PPM1B are responsible for terminating TNFα-induced IKKβ phosphorylation and NF-κB nuclear translocation and activation in the cells.

3.4. PPM1A and PPM1B are required for the down-regulation of TNFα-induced NF-κB dependent IL-6 gene expression

IKKβ is essential in TNFα-induced NF-κB activation and NF-κB-dependent IL-6 expression [28]. To determine the role of PPM1A and PPM1B on the regulation of TNFα-induced IL-6 gene expression, total RNA was extracted from the control, PPM1A and PPM1B knockdown HeLa cell lines treated with or without TNFα for 1 hr. Then RT-PCR was carried out to examine the TNFα-induced IL-6 expression levels in the cells. As shown in Fig. 4A and 4B, TNFα induced a much higher level of the IL-6 expression in the cells with PPM1A and PPM1B knockdown compared to the control cells within 1 hr. These results suggest that PPM1A and PPM1B negatively regulate TNFα-mediated gene expression through inhibiting the TNFα-induced IKKβ phosphorylation and activation.

4. Discussion

IKKβ phosphorylation and activation is an essential step in TNFα-induced NF-κB activation [2]. Phosphorylation of the conserved residues Ser177 and Ser181 within the kinase activation loop is required for IKKβ activation [18]. Following TNFα stimulation, IKKβ is rapidly phosphorylated at Ser177 and Ser181 residues and activated within 5 min. Then IKKβ will be quickly inactivated by dephosphorylation at Ser177 and Ser181 residues suggesting that stringent control of IKKβ phosphorylation and activity is critical for normal TNFα-mediated cellular responses. However, the mechanism of IKKβ dephosphorylation and inactivation following TNFα stimulation to attenuate TNFα-induced NF-κB activation has not been completely defined.

Although several phosphatases have been suggested to be involved in the regulation of IKKβ activity [16,21–23], it is still not clear whether these phosphatases are truly IKKβ phosphatases that downregulate IKKβ activity through the dephosphorylation of IKKβ at the conserved residues Ser177 and Ser181 within the kinase activation loop. Therefore, we decided to take a functional genomic approach to further analyze the mechanism of IKKβ inactivation and identify IKKβ-specific phosphatase(s). In this study, we identify that PPM1A and PPM1B are two major phosphatases involved in negatively regulating IKKβ phosphorylation and activation. We demonstrate that PPM1A and PPM1B are essential to terminate IKKβ-mediated NF-κB activation through binding to the activated form of IKKβ and dephosphorylating IKKβ at the conserved residues Ser177 and Ser181. Our studies suggest that PPM1A and PPM1B function as the IKKβ phosphatases and serve as an important Yin-Yang regulatory mechanism to maintain a delicate balance in TNFα-mediated inflammatory responses.

In this investigation, we found that only PPM1A and PPM1B phosphatase-deficient mutants but not the wildtype are able to pull down the phosphorylated IKKβ, as demonstrated by our co-transfection and immunoprecipitation assays. These data suggest that PPM1A and PPM1B only bind to the phosphorylated IKKβ. In addition, TNFα induces the interaction between IKKβ and PPM1A/PPM1B in a temporary fashion. However, we could not show the TNFα-induced endogenous IKKβ and PPM1A/PPM1B binding possibly due to lack of good antibodies for immunopreciptation of PPM1A and PPM1B. Together, these results are consistent with our prediction that TNFα-induced IKKβ activation is rapidly terminated by IKKβ-phosphatases through physical interaction and dephosphorylation.

Prajapati *et al*. reported that PPM1B negatively regulate IKKβ kinase activity [21]. Consistent with this early report, our studies demonstrate that PPM1B is one of IKKβ phosphatases; meanwhile, we also found that PPM1A is another IKKβ phosphatase. Prajapati *et al.* reported that PPM1B was within the IKK/NEMO complex [21]. However, we failed to observe a strong association between dephosphorylated IKKβ and PPM1B. Instead, we found that the association between IKKβ and PPM1B was transient and IKKβ-phosphorylation dependent. This discrepancy may be explained by the possibility that IKKβ is partially phosphorylated at Ser177 and Ser181 in the co-immunoprecipitation assays reported by Prajapati *et al*. In addition, PPM1B was reported to be involved in the regulation of TAK1 phosphorylation and activation. Purified recombinant PPM1A and PPM1B are able to dephosphorylate IKKβ at Ser177 and Ser181 *in vitro*. This result suggests that phosphorylated IKKβ is a direct target for PPM1A and PPM1B phosphatases. Due to the fact that TAK1 is an upstream activating kinase for IKKβ, it is reasonable to speculate that PPM1B targets these two kinases to inactivate TAK1-IKKβ dependent NF-κB activation. However, it is not clear whether PPM1A also targets TAK1.

PPM1A and PPM1B proteins share 76% amino acid sequence identity [29,30]. Several kinases have been identified to be the substrates for both PPM1A and/or PPM1B including AMPK, CDK2, CDK6, JNKK1, p38 and TAK1 [29,31–34]. Interestingly, PPM1A inhibits TNFα-induced JNK and p38 activation through dephosphorylation of MKK4/JNKK1 and MKK6 as well as p38 [33]. These results suggest that PPM1A and PPM1B may act synergistically to terminate TNFα-induced NF-κB and AP-1 activation in the cells. Interstingly, we observed a strong effect of knockdown of PPM1A or PPM1B expression alone on TNFα-induced IKKβ phosphorylation and activation. These results indicate these two phosphatases do not have a completely functional overlap. Currently, little is known about the regulation of PPM1A and PPM1B function. It is likely that these two phosphatases function in a complex to inactivate IKKβ-mediated NF-κB activation. Further studies are needed to determine the mechanism of PPM1A and PPM1B function in the negative regulation of TNFα-induced IKKβ activation.

Our current studies demonstrate that both PPM1A and PPM1B are two IKKβ phosphatases. However, we can not rule out the possibility that other phosphatases are also involved in IKKβ dephosphorylation and inactivation. Further studies are needed to determine whether other phosphatases are directly involved in the negative regulation of IKKβ activation.

In conclusion, our data provide evidence of the physical and functional interaction between IKKβ and PPM1A and PPM1B. In view of the data presented here and in previous reports, we propose a working model (Fig. 5), in which upon TNFα-induced IKKβ phosphorylation at Ser177 and Ser181, PPM1A and PPM1B phosphatases would bind to the phosphorylated IKKβ. This binding would be a requisite step for PPM1A and PPM1B-mediated dephosphorylation and inactivation of IKKβ as well as the termination of the TNFα-induced NF-κB activation. This report provides the first direct evidence that PPM1A and PPM1B terminate TNFα-induced NF-κB activation through dephosphorylation of IKKβ at Ser177 and Ser181 residues.

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Abbreviations used are

NF-κB
: nuclear factor-κB

IKK
: IκB kinase

PPM1A
: protein phosphatase 1A, magnesium-dependent, alpha isoform

PPM1B
: protein phosphatase 1A, magnesium-dependent, beta isoform

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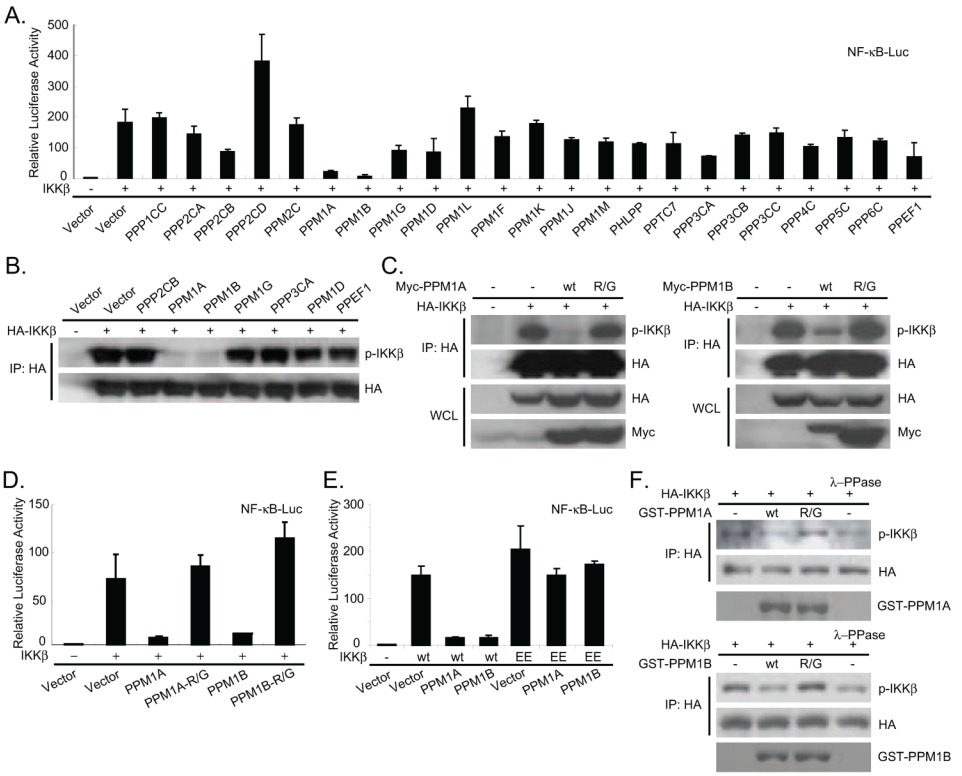

Fig. 1. PPM1A and PPM1B are IKKβ phosphatases

(A) The effect of overexpression of members of serine/threonine phosphatases or the catalytic subunit of polymeric phosphatases on the IKKβ-induced NF-κB activation. HA-IKKβ 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 hrs later and normalized with the *Renilla* activity. Error bars indicate ± standard deviation in triplicate experiments. **(**B**)** The effect of overexpression of members of serine/threonine phosphatases or the catalytic subunit of polymeric phosphatases on the IKKβ phosphorylation at Ser177 and Ser181 within the kinase activation loop. Cell extracts were prepared from HEK 293T cells transfected with expression vectors for HA-IKKβ and different expression vectors for phosphatases as indicated, and analyzed by immunoblotting with the anti-phospho-IKKβ antibody (Ser177/Ser181) (top panel) and reprobed with an anti-HA antibody to detect the expression level of HA-IKKβ (bottom panel). **(**C**)** PPM1A and PPM1B phosphatase activities are required for their effect on IKKβ phosphorylation at Ser177 and Ser181. Cell extracts were prepared from HEK 293T cells transfected with expression vectors for HA-IKKβ and Myc-tagged PPM1A/PPM1B wildtype or phosphatase-deficient versions (R/G), and immunoprecipitated with the anti-HA antibody, then immunoblotted with the anti-phospho-IKKβ antibody (top panel) and reprobed with an anti-HA antibody to detect the expression level of HA-IKKβ (bottom panel). **(**D**)** PPM1A and PPM1B phosphatase activities are required for their effect on IKKβ-induced NF-κB activation. HA-IKKβ 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 wildtype and mutants (R/G) of PPM1A and PPM1B. The relative luciferase activity was measured 36 hrs later and normalized with the *Renilla* activity. Error bars indicate ± standard deviation in triplicate experiments. **(**E**)** IKKβ constitutively active mutant is resistant to the downregulation of IKKβ-mediated NF-κB activation by PPM1A and PPM1B phosphatase activities. Expression plasmid encoding HA-IKKβ wildtype or constitutively

active (EE), along with NF-κB luciferase reporter and *Renilla* luciferase plasmid were co-transfected into HEK 293 cells with empty vector or wildtype PPM1A and PPM1B expression vectors. The relative luciferase activity was measured 36 hrs later and normalized with the *Renilla* activity. Error bars indicate ± standard deviation in triplicate experiments. **(**F**)** Recombinant PPM1A and PPM1B dephosphorylate IKKβ *in vitro*. HA-IKKβ expression vector was transfected into HEK 293T cells and immunoprecipitated with the anti-HA antibody, then treated with 200 μg/ml of wildtype or phosphatase-deficient versions of recombinant GST-PPM1A or PPM1B, or λ-PPase for 30 min before being analyzed by immunoblotting with the anti-phospho-IKKβ antibody and reprobed with an anti-HA antibody to detect the protein level of HA-IKKβ. The λ–PPase was used as a positive control. The recombinant GST-PPM1A and PPM1B proteins used in above assays were detected by Coomassie Blue staining.

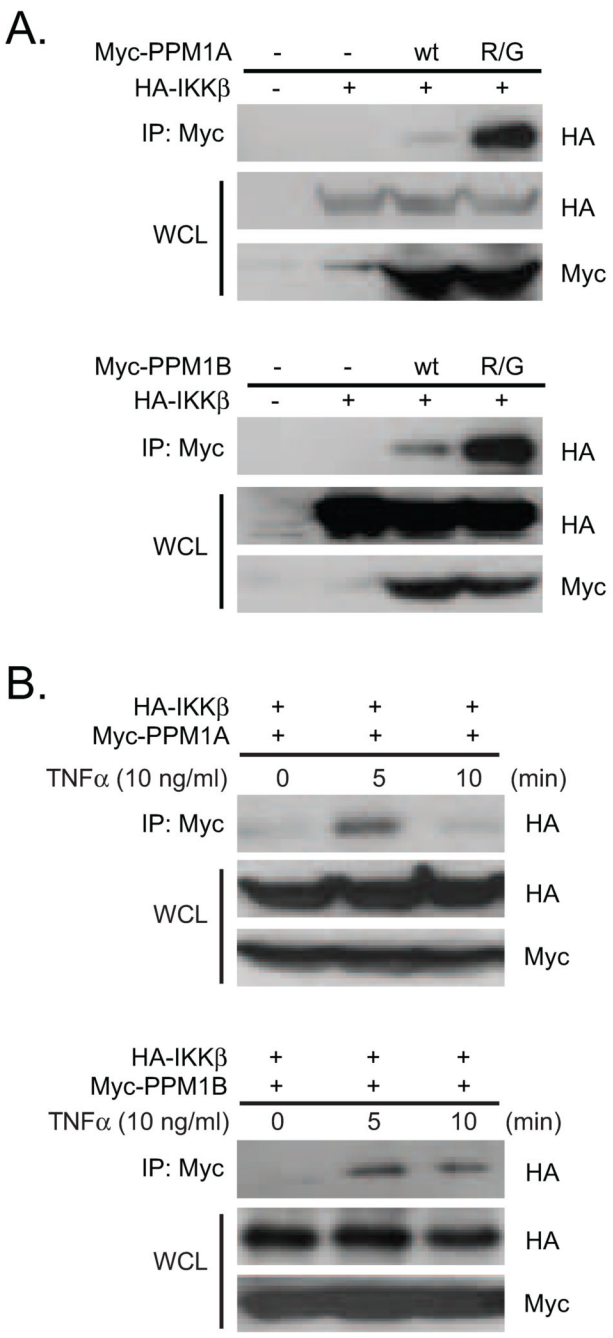

Fig. 2. PPM1A and PPM1B target the phosphorylated IKKβ

(A) PPM1A and PPM1B phosphatase-deficient mutants bind to phosphorylated IKKβ. Cell extracts were prepared from HEK 293T cells transfected with expression vectors for HA-IKKβ and different expression vectors for Myc-tagged phosphatases as indicated, and immunoprecipitated with the anti-Myc antibody, then immunoblotted with the anti-HA antibody. Portions of the WCL were immunoblotted with the anti-HA and anti-Myc antibodies to detect the expression levels of HA-IKKβ and Myc-PPM1A and PPM1B or mutants (R/G) in the cells. **(B)** TNFα induces the association of IKKβ with PPM1A and PPM1B. Expression vectors for HA-IKKβ and Myc-PPM1A or Myc-PPM1B were co-transfected into HeLa cells for 36 hrs, and then cells were either untreated or treated with TNFα (10 ng/ml) for the time

periods as indicated. Myc-PPM1A or Myc-PPM1B in the cell extracts were immunoprecipitated with the anti-Myc antibody, then immunoblotted with the anti-HA antibody to detect the presence of HA-IKKβ. The WCL were immunoblotted with the anti-HA and anti-Myc antibodies to detect the expression levels of HA-IKKβ and Myc-PPM1A and PPM1B in the cells.

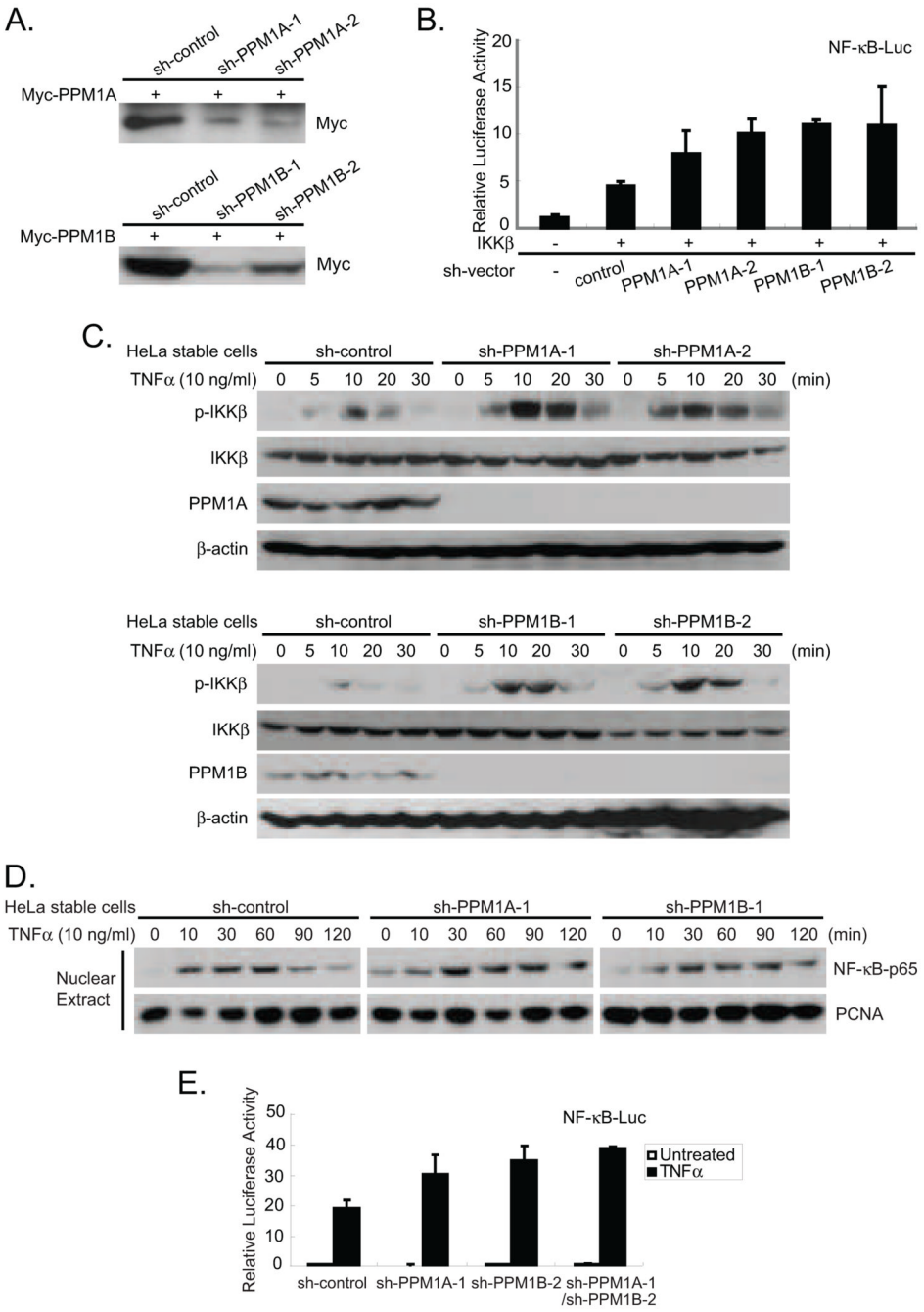

Figure 3. Knockdown of PPM1A and PPM1B expression enhances TNFα-mediated NF-κB activation

(A) Generation of small hairpin RNA (shRNA) expression vectors against PPM1A and PPM1B. Expression vectors for Myc-PPM1A or Myc-PPM1B were co-transfected with shRNA expression vectors against PPM1A and PPM1B into HeLa cells for 48 hrs, and then the cells were lysed and the cell lysates were immunoblotted with the anti-Myc antibody to detect the knockdown effect of these shRNA vectors in the cells. **(**B**)** Knockdown of PPM1A or PPM1B enhances the IKKβ-induced NF-κB activation. HA-IKKβ expression plasmid, NF-κB luciferase reporter plasmid and *Renilla* luciferase plasmid were co-transfected into HeLa cells with shRNA control vector or shRNA expression vectors for PPM1A and PPM1B. The

relative luciferase activity was measured 48 hrs later and normalized with the *Renilla* activity. Error bars indicate ± standard deviation in triplicate experiments. **(**C**)** Suppression of PPM1A and PPM1B expression enhances the TNFα-induced IKKβ phosphorylation in the knockdown cells. HeLa cells with stable expression of sh-control, sh-PPM1A or sh-PPM1B were either untreated or treated with TNFα (10 ng/ml) for the time periods as indicated. Endogenous IKKβ in the cell extracts were immunoblotted with the anti-phospho-IKKβ antibody to detect the level of phospho-IKKβ induced by TNFα, and reprobed with an anti-IKKβ antibody to detect the expression level of IKKβ in the cells. PPM1B and PPM1A knockdown effect by sh-RNA expression were shown by the immunoblotting assay with anti-PPM1A and PPM1B antibodies. β-actin was detected as a loading control. **(**D**)** Knockdown of PPM1A or PPM1B expression enhances the TNFα-induced NF-κB nuclear translocation. The sh-control, sh-PPM1A and sh-PPM1B Hela cell lines were either untreated or treated with TNFα (10 ng/ml) for the time points as indicated and subsequently harvested. The nuclear extracts were prepared and subjected to SDS-PAGE. Nuclear NF-κB-p65 was determined by immunoblotting with an anti-p65 antibody, and PCNA was detected as a loading control. **(**E**)** Knockdown of PPM1A or PPM1B expression enhances the TNFα-induced NF-κB activation. NF-κB luciferase reporter plasmid and *Renilla* luciferase plasmid were co-transfected into HEK 293T cells with sh-control, sh-PPM1A or sh-PPM1B expression plasmids for 30 hrs, and then the cells were either untreated or treated with TNFα (1 ng/ml) for 6 hrs. The relative luciferase activity was measured and normalized with the *Renilla* activity. Error bars indicate ± standard deviation in triplicate experiments.

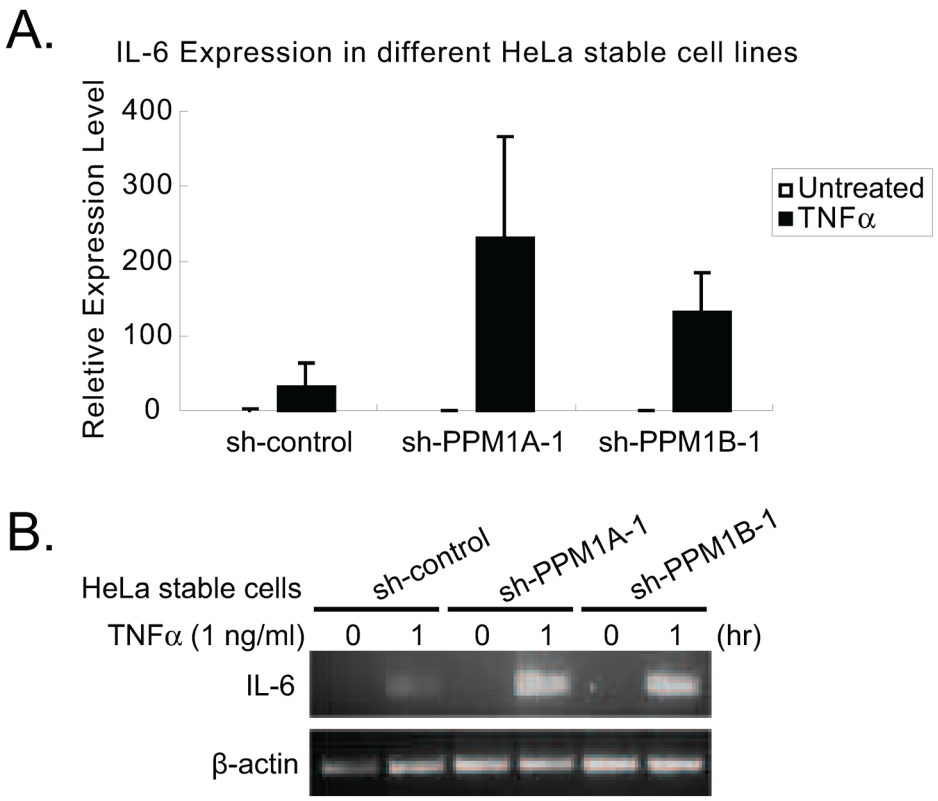

Fig. 4. PPM1A and PPM1B negatively regulate TNFα-mediated IL-6 gene expression

(A) Knockdown of PPM1A or PPM1B expression enhances the TNFα-induced NF-κB dependent IL-6 gene expression. The sh-control, sh-PPM1A and sh-PPM1B cell lines were either untreated or treated with TNFα (1 ng/ml) for 1 hr. Total RNAs from these cells were harvested. IL-6 transcript levels in the sh-control, sh-PPM1A and sh-PPM1B cell lines were measured using quantitative RT-PCR normalized to GAPDH. The data is presented as the average of three separate experiments with standard deviations. **(**B**)** One μg of above total RNA was used to synthesize first-strand cDNA using a reverse transcription kit. The synthesized cDNAs were used as templates for human IL-6 PCR amplification. β-actin PCR amplification was used as a control. The PCR products were resolved in 2% agarose gel.

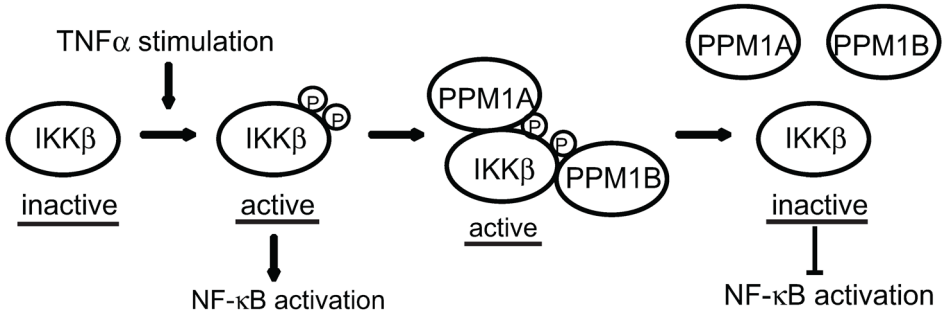

Fig. 5. A working model for IKKβ dephosphorylation and inactivation mediated by PPM1A and PPM1B

TNFα induces IKKβ phosphorylation and activation. PPM1A and PPM1B bind to the phosphorylated IKKβ and subsequently dephosphorylate IKKβ at Ser177 and Ser181 to terminate IKKβ-mediated NF-κB activation.