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PPM1B negatively regulates antiviral response via dephosphorylating TBK1

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ABSTRACT

The production of type I interferon must be tightly regulated and aberrant production of type I interferon is harmful or even fatal to the host. TBK1 phosphorylation at Ser172 plays an essential role in TBK1-mediated antiviral response. However, how TBK1 activity is negatively regulated remains poorly understood. Using a functional genomics approach, we have identified PPM1B as a TBK1 phosphatase. PPM1B dephosphorylates TBK1 in vivo and in vitro. PPM1B wild-type but not its phosphatase-deficient R179G mutant inhibits TBK1-mediated antiviral response and facilitates VSV replication in the cells. Viral infection induces association of PPM1B with TBK1 in a transient fashion in the cells. Conversely, suppression of PPM1B expression enhances virus-induced IRF3 phosphorylation and IFNβ production. Our study identifies a previously unrecognized role for PPM1B in the negative regulation of antiviral response by acting as a TBK1 phosphatase.

1. Introduction

The innate immune system acts as the first line of defense against invasion by microbial pathogens, including viruses, bacteria, and parasites [1–4]. The detection of pathogens occurs through the germline-encoded pattern recognition receptors (PRRs), which at least includes Toll-like receptor (TLR) family, retinoic acid inducible gene I (RIG-I)-like receptor (RLR) family, Nod-like receptor family and C-type lectin receptor (CTR) family [5–9]. Upon PRRs recognize invading viruses, they initiate a series of signaling events leading to robust production of type I interferons (IFNs) and proinflammatory cytokines [10,11]. Type I IFNs further activate downstream signaling pathways that lead to transcriptional induction of a wide range of antiviral genes to cooperatively elicit cellular antiviral response through various mechanisms [10,12]. Although type I interferon is required for viral clearance, the production of type I interferon must be tightly regulated because aberrant production of type I interferon plays a pathological role in autoimmune disorders or could be fatal to the host [13,14].

Activation of RIG-I by double- and single-stranded RNAs or certain viruses leads to its conformational change and then it is recruited to the mitochondrial adaptor protein MAVS (also known as VISA, IPS-1, and Cardif) [15–20]. MAVS is also associated with a scaffolding protein STING (also known as MITA) to recruit the kinase TBK1 to the MAVS-associated signaling complex [21,22]. The C terminus of STING further recruits IRF3 and facilitates the phosphorylation of IRF3 by TBK1. Phosphorylated IRF3 translocates to the nuclear to induce type I IFN production to initiate the antiviral response [23].

TBK1 is a serine/threonine kinase that has been shown to play an essential role in mediating antiviral response [24–26]. Upon viral infection, GSK3β binds with TBK1 and facilitates TBK1 auto-phosphorylation at Ser172 within its kinase activation loop [27]. The activated TBK1 then phosphorylates IRF3/7 and leads to IRF3/7 nuclear translocation as well as IRF3/7-dependent IFN-α/β and RANTES gene expression [28]. However, how the activated TBK1 is negatively regulated by its phosphatase(s) is poorly understood. To our knowledge, there are two phosphatases (SHP2 and SHIP1) that have been reported to negatively regulate IFNß production by targeting on TBK1 [29,30]. SHP-2 inhibits IFNß production by a phosphatase activity-independent mechanism [30]. SHIP1 is a member of the inositol polyphosphate-5-phosphatase (INPP5) family [29]. In this report, we used a functional genomics approach to identify the TBK1 phosphatase(s) by screening a library of serine/threonine phosphatases whose overexpression inhibits TBK1-mediated IRF3 phosphorylation and IFNβ gene expression. Here we present evidence that PPM1B/PP2Cβ functions as a TBK1 phosphatase that dephosphorylates TBK1 at serine 172 and terminates TBK1-mediated IRF3 activation and IFNβ gene expression.

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2. Experimental procedures

2.1. Cell culture and transfection

HEK293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 mg/ml) and glutamine (2 mM). HEK293T and HeLa cells were transfected with FuGene 6 (Roche) and FuGene HD (Roche) respectively according to the manufacturer's recommendation.

2.2. Plasmid construction

Human serine/threonine phosphatase Expression Library was constructed as previously described [31]. The full-length open reading frame of the wild-type PPM1B was subcloned in frame into mammalian expression vector pcDNA3.1 with an N-terminal Myc tag (Invitrogen). The PPM1B (R179G) mutant expression constructs were generated by site-directed PCR mutagenesis (Stratagene) and verified by DNA sequencing. Mammalian expression vector for FLAG-TBK1, FLAG-MAVS and FLAG-RIG-I-Card was obtained from Dr. Paul Chiao (The University of Texas MD Anderson Cancer Center, TX). The retrovirus packing vector Pegpam 3e and RDF vectors were obtained from Dr. Gianpietro Dotti (Baylor College of Medicine). The IFNβ dependent *firefly* luciferase reporter plasmid and pCMV promoter-dependent *Renilla* luciferase reporter plasmid were purchased from Clontech (Mountain View, California). For bacterial expression of PPM1B proteins, cDNAs encoding PPM1B wild-type (PPM1B-wt) and phosphatase-deficient R179G mutant (PPM1B-R179G) were subcloned into pRSET vector (Invitrogen) to generate His-tagged fusion proteins. A pSuper-retro vector (Oligoengine) was used to generate shRNA plasmids for PPM1B. For PPM1B, target sequences are 5′-AATGCAGGAAAGCCATACTGA-3′ (sh-PPM1B-1), and 5′-AACTTCTGGAGGAGATGCTGA-3′ (shPPM1B-2); sequence for sh-Control is: 5′-CTGGCATCGGTGTGGATGA-3′. The authenticity of these plasmids was confirmed by sequencing.

2.3. Antibodies and reagents

Antibodies against HA- and Myc-epitope 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-IRF3 (Ser396) and IRF3 were from Cell Signaling Technology, Inc. (Danvers, MA). Anti-phospho-TBK1 (Ser172) was from BD Biosciences, Inc. Antibody against PPM1B was from Bethyl Laboratories, Inc. (Montgomery, TX). SeV was purchased from Charles River. 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).

2.4. Luciferase reporter gene assays

The luciferase reporter gene assay was performed using a dual luciferase reporter assay system (Promega, Madison, WI) as described previously [31]. Briefly, targeted cells were transiently cotransfected with specific vectors and an IFNβ-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 IFNβ luciferase activity was normalized to *Renilla* luciferase activity. Data are presented as the mean ± standard deviation.

2.5. Quantitative reverse transcription PCR (qRT-PCR) analyses

Total RNAs were prepared using TriZol reagent (Invitrogen) from HeLa sh-Control and sh-PPM1B cells. qRT-PCR was carried out by using 100 ng of total RNA. A volume of 10 μl of 2× 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 OpticonTM 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: IFNβ: 5′-CACAC AGACAGCCACTCACC-3′ and 5′-TTTTCTGCCAGTGCCTCTTT-3′; β-actin: 5′-ACCGCGAGAAGATGACCCAG-3′ and 5′-TTAATGTCACGCACGATTTCC C-3′.

2.6. Generation of stable HeLa cells expressing shRNA targeting PPM1B

The pSuper–PPM1B retroviral construct was transfected into HEK293T cells with retrovirus packing vector Pegpam 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 mg/ml polybrene (Sigma Aldrich). Stable cell lines were established after 10 days of puromycin (2 μg/ml) selection and knockdown efficiency of PPM1B was confirmed by Western blotting.

2.7. 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 g/ml aprotinin, 10 g/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 h with the indicated antibodies. Protein complexes were immunoprecipitated with protein A-agarose (Santa Cruz Biotechnology) for 3 h, 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 Bio-sciences Corp., USA) according to the manufacturer's instruction.

2.8. Purification of His-PPM1B fusion proteins

The bacterial expression plasmids (His-PPM1B-wt and His-PPM1B-R179G) were transformed into *Escherichia 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 h at 30 °C. Bacteria were pelleted and lysed with His extraction buffer (50 mM Tris–HCl, pH 8.5, 100 mM NaCl, 1 mM DTT, 1 mg/ml lysozyme, and 1 mM PMSF) 45 min on ice. The bacteria were sonicated at 4 °C in 1% Sarcosyl (Sigma Aldrich), and 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 His-tagged fusion proteins were collected. A total of 150 μl His-Select TM Nickel Affinity gel (Sigma) was incubated with each bacterial lysate supernatant at 4 °C overnight. 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 (250 mM imidazole, 50 mM Tris–HCl (pH 8.0), 10% glycerol, 300 mM NaCl) and dialyzed in dialyzing buffer (20 mM Hepes (pH 7.9), 150 mM KCl, 0.2 mM EDTA, 20%