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Casein Kinase II Controls TBK1/IRF3 Activation in IFN Response against Viral Infection

Min Du,*1 Jinghua Liu,*1 Xia Chen,* Yadong Xie,* Chuanping Yuan,* Yu Xiang,† Bing Sun,‡ Ke Lan,§ Mingzhou Chen,‖ Sharmy J. James,‖,‡# Yongliang Zhang,‖,‡# Jin Zhong,† and Hui Xiao***

By sensing viral nucleic acids, host innate receptors elicit signaling pathways converging on TBK1-IFN regulatory factor (IRF)3 axis in mediating IFN-αβ induction and defense mechanisms. In contrast, viruses have evolved with diverse immune evasion/interference mechanisms to undermine innate receptor signaling and IFN response. In this regard, approaches enabling host to overcome such immune evasion/interference mechanisms are urgently needed to combat infections by epidemic/pandemic viruses. In this study, we report that protein kinase CK2 serves as a key component controlling TBK1 and IRF3 activation in IFN-inducing TLR, RIG-I–like receptors, and cGAS/STING signaling pathways. Accordingly, knocking down of CK2 expression was able to activate TBK1, whereby eliciting effective host defense mechanisms against hepatitis C virus infection. Taken together, our results identify CK2 as a novel regulator of TBK1 and IRF3 and suggest that targeting CK2 by small molecular inhibitor may be a viable approach to prevent and treat viral infections. The Journal of Immunology, 2015, 194: 4477–4488.

I nnate receptors act at the frontline of virus and host interface by inducing the expression of cytokines, chemokines, and type I IFN, IFN-αβ, whereby initiating antiviral defense

Abbreviations used in this article: BMDM, bone marrow–derived macrophage; HA, hemagglutinin; HCV, hepatitis C virus; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISRE, IFN-stimulated regulatory element; RLR, RIG-I–like receptor; RT, room temperature; SeV, Sendai virus; shRNA, short hairpin RNA; TBB, 4,5,6,7-tetabromobenzotriazole; Trif, Toll/IL-1R domain–containing adapter inducing IFN-β; VSV, vesicular stomatitis virus.

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Address correspondence and reprint requests to Dr. Hui Xiao, Unit of Immune Signaling and Regulation, Institut Pasteur of Shanghai, Chinese Academy of Sciences, 320 Yueyang Road, Life Science Research Building B, Room 211, Shanghai 200031, China. E-mail address: hxuxiao@ips.ac.cn

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cytosolic DNA sensors use mitochondria MAVS (2, 17–19), or endoplasmic reticulum–located STING (20–23), as adaptors to trigger downstream signaling for IFN production, respectively. Despite their differential usage of adaptors, these innate receptor signals eventually converge on the activation of protein kinases TBK1/IKKi, IKKβ, as well as MAPKs, which in turn promote the phosphorylation and activation of IFN regulatory factor (IRF) 3, NF-κB, and AP-1, respectively (1, 3, 4). Despite the role of IKKi in IRF3 activation remaining to be better defined, TBK1 appears to play an indispensable role in IFR3 activation and IFN-αβ induction (24, 25).

Consistent with a critical role of TBK1 and IRF3 in IFN-αβ induction, host has evolved a variety of mechanisms to elegantly control the activation of TBK1/IRF3 axis (26). TBK1 forms complexes with GSK3β, NAP1, and STINBAD (27–30), and becomes associated with TNFR-associated factor 3, NEMO, or STING after viral infection (31). Moreover, recent studies indicate that ubiquitination also plays an important role in TBK1 activation. E3 ligases Mib1/2 and Ndrp1 can assemble K63-type polyubiquitin chains on TBK1 (27, 32), and mutating the lysine 320 to arginine completely abrogated TBK1 activation (33). In addition, TBK1 can be phosphorylated at multiple sites (34), and the phosphorylation of S172 appears to be essential for TBK1 activation (35). Conversely, disrupting the complex formation, ubiquitination, or phosphorylation of TBK1 can attenuate its activation. Deubiquitinating enzyme CYLD or USP2b can dampen TBK1 activation by removing the K63-type polyubiquitin chain (36, 37), and protein phosphatase PPM1B may tamper TBK1 activity through dephosphorylation of S172 (38). Nevertheless, more mechanisms involved in the negative regulation of TBK1 remain to be identified. IRF3 is one of the major downstream substrates of TBK1 in mediating IFN expression. TBK1 mediates the phosphorylation of IRF3, thereby promoting its dimerization, nuclear translocation, and transcriptional activation. Conversely, phosphatases MKP1, MKP5, and PP2A can dephosphorylate IRF3, thus diminishing virus-induced IFN-αβ expression (39, 40). It is conceivable that complex regulation of TBK1/IRF3 phosphorylation by kinases and phosphatases may ultimately determine the magnitude and duration of type I IFN production under viral infection.

Considering type I IFN plays a central role in antiviral defense, it is not surprising that viruses have evolved a diversity of strategies to subvert IFN induction. Influenza can inhibit IFN-αβ induction by compromising the function of RIG-I (41), whereas hepatitis C virus (HCV) almost completely shut down IFN-αβ-inducing signals by disabling adaptors Trif, MAVS, and STING through NS3/4A and NS4B proteins (42–45). Moreover, host proteins SamHD1 and Trex1 can prevent the accumulation of reverse-transcribed DNA (6, 7, 9, 44, 46, 47), thus facilitating HIV to evade the recognition by DNA sensors such as cGAS and IFI16. Therefore, strategies capable of overcoming such viral immune evasion/interference mechanisms may rejuvenate IFN induction and host defense against these viruses.

CK2 is a multifunctional serine/threonine kinase composed of two catalytic subunits (CK2α or α′) and two regulatory subunits (CK2β), and it plays a broad role in cell growth, survival, and cancer (48). Because CK2 is involved in a variety of physiological and pathological conditions, small chemical inhibitors of CK2, such as 4,5,6,7-tetrambromobenzotriazole (TBB), have been widely tested in treating neurologic diseases, cardiovascular diseases, and cancers (49, 50). Recent studies suggest that CK2 is also involved in the immune regulation by phosphorylating NF-κB (51–53) and RIG-I (54). To our knowledge, in this study, we first demonstrate that protein kinase CK2 functions as a key regulator of TBK1 and IRF3, controlling IFN-αβ response at steady state and under viral infection. Notably, compromising CK2 function by short hairpin RNA (shRNA) or small molecule inhibitor TBB led to hyperactivation of TBK1 and elevation of IFN-αβ response, thereby creating an antiviral state in a diversity of cells. As many viral IFN-αβ–dampening tactics impinge on innate receptors or adaptors, it is conceivable that activating TBK1 by small molecules such as TBB may be a viable approach to overcome viral immune evasion/interference mechanisms, and revive IFN response in patients suffering chronic infections from viruses like HCV, hepatitis B virus, and HIV.

Materials and Methods

Cells and reagents

Bone marrow cells were isolated from 6– to 8-wk C57BL/6 mice purchased from Shanghai Laboratory Animal Center (Shanghai, China). All the procedures were conducted in compliance with a protocol approved by the Institutional Animal Care and Use Committee at Institute Pasteur of Shanghai. Bone marrow cells were cultured in RPMI 1640 medium supplemented with 30% L929-conditioned medium (containing M-CSF), as well as 10% FBS (HyClone) for 5–7 d. Murine macrophage RAW 264.7, fibroblast 3T3, and human HeLa, 293 T, and human Hela (American Type Culture Collection) were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 2 mM t-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. LPS, poly(I:C), and poly(dA:dT) were from Invivogen, and DMXAA was purchased from Selleck Chemicals.

Plasmids and primers

The cDNA for CK2α was amplified from total RNAs isolated from C57BL/6 bone marrow cells by forward primer 5′-TGGAATTCCAGAC-ATGTCGGGACCCGTG-3′ and reverse primer 5′-TATCGCGCCGCT-TACTGCGTACGCCGACGACG-3′. CK2β was cloned into pIP-Flag and pCDH vectors through EcoRI and NotI. The shRNA for CK2α was generated by overlapping approach using forward primer 5′-ACGAGGTGTTGATGATTCTCCATACACATGACACTCTTAAGTATTCTCGAG-3′ and reverse primer 5′-GAGCAGCTAGCTCTCAATGGGCAACAGCCACC-3′. The shRNA for ppp2ca was generated from RNAs from C57BL/6 bone marrow cells by forward primer 5′-TTTGGATCCATGGAGGAGAAGTGTTTGACGACACTCTTAAGTATTCTCGAG-3′ and cloned into pIP-HP vector through BamHI and XbaI. The cDNA for IRF3 was amplified from total RNAs isolated from B6 bone marrow cells by forward primer 5′-AGAGTTATTCAATGGAACCCCAAGCACC-3′ and reverse primer 5′-AAAGCAGGGCCGCTCAGCTCTCCCCAGGCCGG-3′. The shRNA for ppp2ca was generated by overlapping approach using forward primer 5′-ATACCTCGAGCTTCCACATGACACGTCG-3′ and reverse primer 5′-CATACCTCGAGCTTCCACATGACACGTCG-3′ and cloned onto pIP-HP vector by BamHI and XbaI. The vectors pIP-Flag and pIP-HP were gifts of B. Li, (Institut Pasteur of Shanghai) and described previously (55), shRNA-expressing vector plKO.1 was purchased from Addgene. The shRNA for CK2α was generated by forward primer 5′-CCGGCGCCAGTGTCTCTCAGATATCGGAGAAGAAGTATTCTCGAG-3′ and reverse primer 5′-GGGAGTTGGTTGCTGTGTCTGGATCAATTGCAATGACACTCTTAAGTATTCTCGAG-3′. The shRNA for CK2β was generated by forward primer 5′-CCGGCGCCAGTGTCTCTCAGATATCGGAGAAGAAGTATTCTCGAG-3′ and reverse primer 5′-GGGAGTTGGTTGCTGTGTCTGGATCAATTGCAATGACACTCTTAAGTATTCTCGAG-3′. The shRNA for ppp2ca was generated by forward primer 5′-CCGGCGCTTCCACATGACACGTCG-3′ and reverse primer 5′-AATTCTGAAGTTTGGATCCATGGAGGAGAAGTCGCTCTCGAG-3′.
1.5 mM MgCl₂, 1 mM EDTA, supplemented with protease inhibitors and phosphatase inhibitors, as described above) to make crude nuclear fraction. Crude cytosolic and nuclear fractions were further centrifuged at 13 Krpm for 15 min to remove debris, and the supernatants were collected as cytosolic and nuclear fractions.

**Immunoprecipitation and immunoblotting**

For immunoprecipitation, whole-cell lysates were incubated with 2–5 μg Abs overnight, followed by incubation with 10–20 μl protein A–Sepharose 6MB beads (GE Healthcare Life Sciences) for 1 h at 4°C. The immunoprecipitates were washed three times with wash buffer (50 mM Tris [pH 8.0], 300 mM NaCl, 1% Triton X-100, 1 mM EDTA). Proteins were eluted with 2× SDS loading buffer by boiling for 10 min. Protein samples were resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membrane, which was then probed by Abs. Abs against p-pIkBa (Ser53, 2859), p-p38 (Thr180/Tyr182, 9211), p-JNK (Thr183/Tyr185, 4668), JNK (9252), hemagglutinin (HA: 3721), p-TBK1 S172 (5483), TBK1 (3504), p-IRF3 S396 (4947), IRF3 (4310), CK2α (2656), and p-STAT1 (Ser790, 9145) were from Cell Signaling Technology. Abs against Erk (SC-93), p-ERK (SC-728), Tubulin (SC-5274), LaminB1, and histone were from Santa Cruz Biotechnology. Anti–PP2-Ca was from BD Biosciences; anti-p65 S529 (ab47395) was from Abcam; and anti-GAPDH (AP0063) was from Bioworld. Abs to FLAG (F3165) or FLAG M2 beads were purchased from Sigma-Aldrich.

**ELISA**

Cells were stimulated by various viruses or ligands for 6 or 24 h. Supernatants were harvested, and the secreted amounts of IFN-α, IFN-β, CXCL1, CXCL10, or TNF-α were measured by ELISA kits (eBioscience; R&D Systems), according to manufacturers’ recommendations.

**RNA preparation and real-time PCR**

RNAs were extracted from bone marrow–derived macrophages (BMDMs), Raw 264.7, L929, and HuH7.5 cells using TRIzol (Invitrogen), according to the manufacturer’s instruction. cDNA was reversely transcribed from 1 μg total RNA by SuperScript III First Strand Synthesis Kit (Invitrogen). Real-time PCR was carried out with PrimeScript RT reagent kit (Takara) on ABI 7900HT Fast Real-Time PCR System. Relative expression of target genes was quantitatively normalized against the expression of Gapdh using ΔΔct method. Primer sequences are shown in Table S1. Relative expression of target genes was calculated by the 2⁻ΔΔct method. Primer sequences are shown in Table S1.
LANOVA and presented as means ± SEM. Brieﬂy, the supernatants harvested from VSV-infected cells were serial diluted and then added into confluent HEK 293 cells cultured on 24-well plates. One hour postinfection, supernatants were removed and cells were overlaid by 3% methylcellulose. Three days later, overlay was removed and cells were ﬁxed with 4% formaldehyde for 20 min and then stained with 0.2% crystal violet. Plaques were counted, and viral titers (PFU/ml) were calculated accordingly. HSV-1 plaque assay was conducted similarly in Vero cells (57).

HCV JFH-1-GFP chimeric viruses were propagated in HuH7.5 cells. The ﬂuorescence form unit of HCV JFH-1-GFP viruses was measured, as reported previously (58). Brieﬂy, supernatants from infected HuH7.5 cells were serial diluted in complete DMEM, and added into HuH7.5 cells (1 × 10^3 cells/well) seeded on 8-well chamber slides for 4 h. The inoculums were removed, and cells were cultured in 200 μL fresh medium for 3 d. Cells were then ﬁxed in methanol:acetone (1:1) solution for 9 min at room temperature (RT) and stained by anti-core Ab (1:300) for 2 h at RT. Subsequently, cells were incubated with Alexa 488–conjugated secondary Ab diluted (1:100 in PBS) for 1 h at RT. Slides were then examined under ﬂuorescence microscope, and foci of infection were counted.

**Statistical analysis**

Two-way ANOVA with Bonferroni posttest and two-tailed unpaired t test with 95% conﬁdence interval were used for statistical analysis, and a p value < 0.05 was considered signiﬁcant.

**Results**

**CK2 negatively regulates HSV-1–induced IFN response**

As phosphorylation plays an important role in the regulation of virus-sensing innate receptor signaling, we sought to systemically identify protein kinases involved in the regulation of virus-induced type I IFN induction. By screening a library of small chemical compounds targeting protein kinases, we found that inhibition of kinases ATM, PLK, CK1, TPL2, ERK, PI3K, and IKKβ/TBK1 diminished IFN-β expression, whereas inhibition of CK2 by TBB or GSK3 by SB216763 elevated IFN-β expression induced by HSV-1 (Supplemental Fig. 1). CK2 is a multifunctional serine/threonine kinase that can regulate cell growth, apoptosis, and inﬂammation (48). Although CK2 can function as a positive regu-

FIGURE 2. CK2 negatively regulates IFN response induced by RNA viruses. (A and B) Murine ﬁbroblast L929 cell lines transduced by lentiviral pLKO.1 vector expressing either scrambled shRNA or ck2α-specific shRNA (sh-ck2α) were infected by SeV (multiplicity of infection: 10) or VSV-GFP (multiplicity of infection: 1) for 6 h, followed by RNA preparation through TRIzol. Quantitative real-time PCR was conducted to measure the relative expression levels of Ifna, Ifnb, Cxcl10, Mx1, and Mx2, respectively. (C) L929 cell lines were infected by VSV-GFP (multiplicity of infection: 1) for 6 h, and supernatants were collected for ELISA. (D) sh-scrambled or sh-ck2α-transduced L929 cells were infected by VSV-GFP (multiplicity of infection: 0.01 or 0.1) for 24 h. Supernatants were collected, and viral titers were measured by plaque assay, as described in **Materials and Methods**. Data are analyzed by two-way ANOVA and presented as means ± SEM. Similar results were obtained from two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
To validate the negative role of CK2 in IFN regulation, we used shRNA approach to specifically knock down the expression of CK2α, the predominant CK2 isoform in Raw 264.7 cells (59, 60). Upon lentiviral transduction, stable pools of Raw cells expressing either control or CK2α-specific shRNAs were created by puromycin selection, and the expression levels of CK2α protein were efficiently knocked down by two different shRNAs (sh-ck2α and sh-ck2α#) (Fig. 1A). Following HSV-1 infection, two stable pools of CK2α–knocking-down Raw cells exhibited elevated expression of Ifna/b mRNA (Fig. 1B, 1C). Consistently, a group of ISGs, such as Mx1, Mx2, and Cxcl10, was highly induced in CK2α–knocking-down Raw cells. It is interesting to note that knocking down of CK2α expression also had similar effect on IFN expression at steady state (Fig. 1B, 1C). Furthermore, HSV-1 titers were diminished in CK2α–knocking-down Raw cells (Fig. 1D), which is consistent with elevated IFN response in these cells (Fig. 1B). These results demonstrate that CK2 may function as a negative regulator of IFN responses under steady state and HSV infection.

To further address the role of CK2 kinase activity in the regulation of HSV-induced IFN response, we made a kinase-inactive mutant of CK2α (K68M) and generated Raw cell lines that stably express wild-type or kinase-inactive mutant of CK2α (Fig. 1E). Interestingly, exogenous expression of CK2α resulted in diminished Ifna/b and Cxcl10 expression (Fig. 1F), an effect opposite to that caused by knocking down of CK2α (Fig. 1B, 1C). Conversely, exogenous expression of the kinase-inactive mutant of CK2 failed to have a significant impact on HSV-induced IFN response in Raw cells (Fig. 1F), indicating a critical role of CK2 kinase activity in the regulation of IFN-α/β expression.

**CK2 is involved in RNA virus-induced IFN response**

Next, we investigated whether CK2 is involved in antiviral response against a broad range of viruses. Upon infection by SeV, ck2α–knocking-down L929 cells also expressed much more Ifna, Ifnb, Cxcl10, Mx1, and Mx2 mRNA than control cells (Fig. 2A). Furthermore, elevated IFNa/β and Cxcl10 were detected in vesicular stomatitis virus (VSV)–infected ck2α–knocking-down L929 cells by RT-PCR (Fig. 2B) and ELISA (Fig. 2C). More importantly, ck2α–knocking-down cells were highly resistant to VSV infection. Upon infection by low titers of VSV-GFP (multiplicity of infection: 0.01 and 0.1), very few GFP-positive cells were detected by fluorescence microscopy 24 h postinfection (Supplemental Fig. 2). Consistently, many less viral particles were detected in the supernatants collected from ck2α–knocking-down cells (Fig. 2D), indicating that elevated IFN response might have improved cellular control of VSV infection. Therefore, we identified CK2 as a negative regulator critically controlling IFN response to viral infections.

**CK2 negatively regulates TLR3/4-induced TBK1/IRF3 activation**

As described above, we identified CK2 as a key regulator of HSV-induced IFN expression, which has been attributed to TLR3/Trif and cGAS/STING pathways (61, 62). We first examined whether CK2 plays a role in Trif-mediated IFN response. BMDMs transduced by lentiviral sh-ck2α were treated by TLR3 ligand poly(I:C)
or TLR4 ligand LPS; the expression of IFN-β and ISGs was assessed by ELISA and RT-PCR, respectively. By ELISA, we detected elevated IFN-β and CXCL10 in ck2α–knocking-down BMDMs stimulated by poly(I:C) or LPS for 24 h (Fig. 3A). By RT-PCR, Ifnb, Cxcl10, and Mx1/2 mRNAs were also induced greater in ck2α–knocking-down BMDMs stimulated by poly(I:C) or LPS for 4 h (Supplemental Fig. 3). Conversely, TNFa, IL-6, and Cxcl1/mRNAs were even reduced in ck2α–knocking-down BMDMs (Supplemental Fig. 3). These results indicate that the negative regulatory function of CK2 is restricted to IFN response in TLR3/4 signaling.

Next, we sought to determine how CK2 might influence TLR-triggered signaling events. Knocking down of ck2α led to reduced p-IκB in LPS-stimulated BMDMs (Fig. 3B), consistent with a positive role of CK2 in NF-κB activation (51, 53). Conversely, the phosphorylation and activation of MAPKs p38, ERK, and JNK were unaffected. Decreased NF-κB activation was well correlated with diminished Cxcl1 and IL-6 mRNA expression in these cells (Supplemental Fig. 3). Remarkably, phosphorylation of TBK1 (p-S172) was pronounced elevated in CK2α–knocking-down BMDMs stimulated by LPS or poly(I:C) (Fig. 3C, 3D). Accordingly, IRF3 phosphorylation on S396 was greater in CK2α–knocking-down BMDMs (Fig. 3C, 3D). In accordance with greater induction of IFN-β in CK2α–knocking-down BMDMs, p-STAT1 was markedly induced in these cells following LPS and poly(I:C) treatment (Fig. 3C, 3D). These results revealed a novel role of CK2 in regulating TBK1 and IRF3 activation in TLR3/4 signaling.

CK2 negatively regulates STING-mediated TBK1/IRF3 activation

Transfection of poly(I:C) or poly(dA:dT) into L929 cells induced robust IFN-α/β expression by engaging MAVS or STING pathway, respectively. Interestingly, the induction of IFNα/β, as well as
ISGs *Mx1*, *Mx2*, and *Ccl10* by poly(I:C) and poly(dA:dT), was considerably upregulated in CK2α–knocking-down L929 cells (Fig. 4A). Furthermore, DMXAA, an agent that can directly bind to murine STING and mimic the action of cytosolic dsDNA (63, 64), also induced stronger IFN response in CK2α–knocking-down L929 cells (Fig. 4A). These results indicate that CK2 is involved in the regulation of IFN response in intracellular RNA- and DNA-sensing pathways as well.

Next, we examined whether CK2 also involves the regulation of TBK1 and IRF3 activation in STING-mediated signaling. Upon stimulation by DMXAA, STING becomes activated, leading to the phosphorylation of TBK1 and IRF3. Notably, we observed marked accumulation of phosphorylated TBK1 in the nucleus of L929 cells stimulated by DMXAA (Fig. 4B). Consistently, nuclear translocation of IRF3 was evidently upregulated in CK2α–knocking-down L929 cells (Fig. 4B). Furthermore, p-STAT1, presumably induced by autocrine IFN-α, was also elevated in CK2α–knocking-down L929 cells (Fig. 4B). Similarly, TBK1 and IRF3 phosphorylation was elevated in the nuclear compartment of CK2α–knocking-down cells before and after DMXAA stimulation (Fig. 4C). Whereas STING-mediated signaling initiates TBK1 activation and IRF3 phosphorylation in the cytoplasm, our observations show that p-TBK1 is also present in the nucleus, thus suggesting complex mechanisms are involved in the regulation of TBK1/IRF3 axis.

Together, we identified CK2 as a novel regulator of TBK1 and IRF3 activation in multiple IFN-inducing innate receptor signals. **CK2 regulates TBK1 and IRF3 activation through intermediate phosphatase**

Next, we attempted to dissect the molecular mechanism by which CK2 regulates TBK1 and IRF3 activation. By conducting luciferase reporter assay, we found that whereas CK2 had some inhibitory effect on RIG-I-, MAVS-, STING-, or Trif-mediated IFN-stimulated regulatory element (ISRE)–luciferase expression, its inhibitory effect on TBK1 and IRF3 was more prominent (Fig. 5A), suggesting that CK2 may exert its effect on TBK1 and/or IRF3. Moreover, CK2 did not have any impact on IRF3 S396D, a constitutive active mutant of IRF3 that mediated ISRE-driven luciferase expression (Fig. 5A), suggesting that CK2 may be involved in the regulation of IRF3 phosphorylation. However, we were unable to detect the physical association between CK2 and TBK1 or IRF3 by immunoprecipitation (Supplemental Fig. 4A, 4B). These results prompted us to propose that CK2 may downregulate TBK1 and IRF3 phosphorylation through intermediate protein phosphatase.

Among >300 substrates identified for CK2, we noticed that PP2A and PTEN are well-defined protein phosphatases (48, 65).

**FIGURE 5.** CK2 regulates IRF3 phosphorylation through phosphatase PP2A. (A) Plasmids expressing ISRE-firefly luciferase or Renilla luciferase were cotransfected with plasmid expression Trif, MAVS, STING, TBK1, IRF3, IRF3 S396D, or IRF7 along with plasmid expressing CK2α into HEK293T cells by calcium-phosphate method. Transfected cells were harvested 36 h later, and luciferase activities were measured by dual-luciferase assay kit (Promega), according to manufacturer’s instruction. The data are presented as the relative firefly luciferase/Renilla luciferase ratio. (B) L929 cells were either untreated or treated by DMXAA (100 μg/ml) for 3 h. Cell lysates were immunoprecipitated by anti-IgG or anti-CK2α, respectively. Immunoprecipitates were probed by anti–PP2A–Ca and anti–CK2α sequentially. (C) sh-scrambled or sh-ppp2ca–transduced L929 cells were either untreated or treated by DMXAA (100 μg/ml) for 3 h. Whole-cell lysates were prepared and immunoprecipitated by anti-IgG or anti-CK2α. Immunoprecipitates absorbed on protein A beads were used for in vitro PP2A phosphatase assay using phospho-peptide KRpTIRR as substrate (EMD Millipore), according to manufacturer’s instruction. Western blots were carried out to examine the phosphorylation of TBK1 and IRF3, respectively. (D) sh-scrambled or sh-ppp2ca–transduced L929 cells were stimulated by DMXAA for 6 h. Whole-cell lysates were harvested and resolved by 8% SDS-PAGE, and were then transferred onto a polyvinylidene difluoride membrane. Western blots were carried out to examine the phosphorylation of TBK1 and IRF3, as well as the expression levels of TBK1, IRF3, and PP2-Ca, respectively. (E) Cell lysates from Raw cells expressing either pLP-Flag, pLP-Flag-PP2A, or pLP-Flag-PP2Amut (L199P, phosphatase dead) were bound to anti-Flag agarose beads for 3 h at 4˚C to purify recombinant PP2A proteins. p-IRF3 proteins were purified from Raw264.7 cells transfected with pLP-HA-IRF3 and stimulated with LPS (100 ng/ml) for 1 h using anti-HA agarose beads. In vitro dephosphorylation assay was performed by incubating equal volumes of purified p-IRF3 with Flag-PP2A or Flag-PP2Amut for 2 h at 37˚C in phosphatase assay buffer, as described previously (69). Following the termination of dephosphorylation assay, p-IRF3 was measured by Western blotting. Above experiments were repeated at least once, and the representative data are shown. *p < 0.05, ***p < 0.001.
We therefore tested whether these two phosphatases are involved in the regulation of TBK1 and/or IRF3 phosphorylation. Despite the fact that PTEN does not interact with CK2 (data not shown), PP2A formed complexes with CK2, and DMXAA stimulation diminished such complex formation (Fig. 5B). Through phosphatase assay, we found that the phosphatase activity of PP2A was greatly reduced in ck2a–knocking-down cells, indicating that CK2 positively regulates PP2A activity (Fig. 5C). Furthermore, we found that PP2A was able to interact with both TBK1 and IRF3 (Supplemental Fig. 4C, 4D). We therefore knocked down the expression of ppp2ca, which encodes PP2-Ca, the catalytic subunit of PP2A, to investigate the role of PP2A in TBK1 and IRF3 activation. Interestingly, knocking down of ppp2ca expression only led to elevated phosphorylation of IRF3, but not TBK1 (Fig. 5D), suggesting that PP2A may be directly involved in the regulation of IRF3 phosphorylation. By dephosphorylation assay, we found that PP2A was able to dephosphorylate IRF3 on S396, a process that depends on its phosphatase activity (Fig. 5E).

In addition, we found that DMXAA- and VSV-induced IFN responses were elevated in ppp2ca–knocking-down cells (Fig. 6A, 6B), indicating that PP2A negatively regulates IFN response. Furthermore, whereas blockade of CK2 activity by TBB drastically elevated DMXAA-induced IFN response in wild-type cells, TBB only had a marginal effect on elevating DMXAA-induced IFN response in ppp2ca–knocking-down cells (Fig. 6C). Collectively, these results suggest that CK2 may influence IFN response in part through the intermediate phosphatase PP2A.

**Blockade of CK2 activity by TBB elevates IFN response**

As shown above (Figs. 1E, 6C), the kinase activity of CK2 may play a critical role in the regulation of IFN response; we therefore tested whether inhibition of CK2 activity can influence TBK1 and IRF3 activation. TBB has been demonstrated to be a specific inhibitor for CK2 (50) and can efficiently inhibit CK2-mediated phosphorylation of NF-κB p65 on S529 (51, 52). In TBB-treated L929 cells, we first examined p65 phosphorylation and found the p65 phosphorylation on S529 was drastically reduced by TBB, indicating that CK2 activity was efficiently blocked (Fig. 7A). Remarkably, phosphorylation of TBK1 was pronounced induced in L929 cells by TBB in a time- and dose-dependent manner (Fig. 7A). Although p-IRF3 was too weak to detect by straight Western blotting, we were able to see p-IRF3 in the anti-IRF3 immunoprecipitates after TBB treatment (Fig. 7B). Consistently, TBB treatment elevated the expression of IFNα/β, Mx1/2, and Cxcl10 (Fig. 7C). Compared with untreated cells, TBB-pretreated cells exhibited considerably fewer VSV-GFP–positive cells and produced markedly reduced VSV particles (Fig. 7D).

We further examined whether the antiviral effect of TBB can be extended to human cells. Upon pretreatment by TBB, phosphorylation of TBK1 was evidently detected in both cytosolic and nuclear compartments of human HEK293T cells (Fig. 7E). Consistently, p-STAT1 and IFN responses were also elevated in HEK293T cells predisposed to TBB (Fig. 7E, 7F). Remarkably, TBB-treated HEK293T cells demonstrated improved resistance to viral infection (Fig. 7G). These results demonstrate that blockade of CK2 by TBB alone was sufficient to activate TBK1, which can elicit IFN response to control viral infection.

**Blockade of CK2 activity by TBB can be an effective treatment for HCV infection**

HCV is one of the most notorious viruses capable of dampening host IFN responses through its NS3/4A and NS4B proteins. Be-
cause NS3/4A and NS4B primarily target MAVS, Trif, or STING, respectively, which are upstream of TBK1, we reasoned that blocking CK2 by TBB might be able to overcome such immune interference tactics. To test this hypothesis, we first pretreated Huh7.5 cells [a HCV-permissive cell line expressing a mutant form of RIG-I (66)] by TBB for 24 h, and then infected them by HCV for 48 h. TBB treatment led to reduced phosphorylation of NF-κB p65 on S529, indicating that CK2 kinase activity was blocked (Fig. 8A). Consistently, blockade of CK2 by TBB also resulted in elevated p-TBK1 and p-STAT1 in Huh7.5 cells (Fig. 8A). Remarkably, compared with HCV infection, TBB pretreatment led to much stronger IFN response (Fig. 8B). Moreover, by measuring HCV particles released from infected cells, we found that HCV infection and replication were considerably inhibited by TBB pretreatment (Fig. 8C).

Next, we investigated whether TBB can inhibit HCV replication postinfection. Huh7.5 cells infected by HCV (multiplicity of infection: 0.1 and 0.01) for 24 h were treated by TBB for another 48 h, followed by assessment of IFN response and viral titers. Similar to pretreatment, posttreatment by TBB also significantly elevated IFN response, which was well correlated with prominent reduction of viral titers (Fig. 8D, 8E). It is also noteworthy that IFN response was highly induced in TBB-treated cells even though HCV replicated inefficiently in these cells. This phenomenon is consistent with the notion that TBB-induced TBK1 activation is not dependent on upstream signaling triggered by viral RNA. These data indicate that blocking CK2 activity by TBB can be a viable approach to overcome the immune interference mechanisms posed by viruses such as HCV.

### Discussion

By recognizing viral PAMPs, innate receptors play a critical role in the induction of IFN and orchestration of innate and adaptive immune responses. Despite the fact that a diversity of IFN-inducing innate receptors has been identified, the mechanisms by which these receptors induce adequate, yet effective immune response against specific viruses have been much less understood. Whereas rapid and efficient IFN response is desirable for the control of viral infection, it can also be detrimental when dysregulated. As expected, complex regulatory mechanisms are involved in the regulation of IFN-inducing signaling. In this study, we uncovered that CK2 plays a crucial role in the regulation of TBK1/IRF3 activation, whereby controlling IFN response against a variety of viruses. Despite the fact that phosphorylation has been widely regarded as an essential step for TBK1 activation, the reciprocal regulation of its reversible phosphorylation has been very much understudied. Besides TBK1 itself, none of the other kinases has been linked to phosphorylation of TBK1/IRF3. To our knowledge, our study first uncovered that protein kinase CK2 is involved in TBK1 and IRF3 phosphoregulation, thus revealing another layer of regulation on IFN induction and providing new insight into the understanding of phosphoregulation of TBK1/IRF3 axis.

CK2 is a pleiotropic Ser/Thr protein kinase involved in a wide range of physiological and pathological processes, including cell growth/survival, cancer, inflammation, and viral infection. Although being widely considered as constitutively active, CK2 activity can be further upregulated by stress or viral infection. Previous studies have demonstrated that CK2 can be involved in the regulation of inflammatory or stress response by phosphorylating...
IKK and NF-κB. Consistent with these reports, our study demonstrated that CK2 plays a positive role in the regulation of NF-κB activation and proinflammatory cytokine production at an early stage of TLR signaling (Figs. 3B, 7A, 8A, Supplemental Fig. 3). However, the positive regulation on NF-κB cannot be attributable to the negative effect of CK2 on IFN response demonstrated by this study. In addition, a recent report suggests that CK2 may be able to promote RIG-I phosphorylation (54), whereby negatively regulating RIG-I–mediated signaling. It is highly possible that the negative effect of CK2 on both RIG-I and TBK1 might have contributed to elevated IFN response in CK2–knocking-down cells stimulated by SeV, VSV, or transfected poly(I:C), which presumably engage RIG-I signaling (Figs. 2A, 2B, 4A). However, elevated IFN response in CK2–knocking-down cells stimulated by TLR ligands, poly(dA:dT)/DMXAA, or HSV should be predominantly attributed to its action on TBK1/IRF3 rather than RIG-I (Figs. 1, 3A, 4A). It is worth noting that HCV-permissive Huh7.5 cells express a nonfunctional mutant of RIG-I (66); thus, the effect

FIGURE 8. Blockade of CK2 by TBB prevents HCV infection. (A) Huh7.5 cells (5×10^4/ml) were either untreated or treated by TBB (20 or 100 μM) for 6 and 12 h, respectively. Whole-cell lysates were prepared, and immunoblotting was conducted to detect the phosphorylation of p65, TBK1, and STAT1, respectively. (B and C) Huh7.5 cells (5×10^4/ml) were pretreated by 100 μM TBB for 24 h, followed by infection of HCV-GFP (multiplicity of infection: 0.01 or 0.1) for 48 h. (D and E) Huh7.5 cells were infected by HCV-GFP first, 24 h postinfection, and 100 μM TBB was added into infected cells and incubated for another 48 h. Subsequently, RNAs were extracted; the relative expression levels of Ifna/β and Mx1/2 were measured by real-time PCR; and supernatants were collected for measuring HCV titers, as described in Materials and Methods. Data are analyzed by two-tailed unpaired t test and presented as means ± SEM. Similar results were obtained from two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
of TBB in these cells cannot be attributed to RIG-I either (Fig. 8). Taken together, despite the fact that CK2 can act on multiple substrates in innate receptor signaling, our study nevertheless strongly argues that CK2-regulated TBK1/IRF3 activation plays a crucial role in controlling IFN response against various viruses.

How CK2 regulates TBK1 and IRF3 phosphorylation remains an important, but less understood question. Despite the fact that CK2 diminished TBK1 or IRF3-driven IFN response (Fig. 5A), we were unable to detect the physical association of CK2 with TBK1 or IRF3 (Supplemental Fig. 4A, 4B). These results implicate that CK2 may downregulate TBK1 and IRF3 phosphorylation through intermediate protein phosphatase. Among >300 substrates identified for CK2, PP2A, and PTEN are well-defined protein phosphatases (48, 49, 65). However, we only detected physical interaction of CK2 with PP2A, but not PTEN. Furthermore, we found that CK2 can elevate PP2A phosphatase activity in STING-mediated signaling (Fig. 5C), corroborating previous reports suggesting that CK2 can phosphorylate and activate PP2A (57, 67). Moreover, PP2A is involved in the negative regulation of virus-induced IFN response (Fig. 7A, 7B), and the inhibitory effect of CK2 on IFN response was significantly impaired in PP2A-knockdown cells (Fig. 7C), identifying PP2A as a key downstream target of CK2 in regulating IFN response. However, knocking down of PP2A mainly elevated the phosphorylation of IRF3, rather than TBK1 (Fig. 5D), suggesting that CK2 may regulate TBK1 activation through molecules other than PP2A. However, the downstream target by which CK2 regulates TBK1 phosphorylation remains to be identified. Despite the fact that phosphatase PPM1B has been shown to be involved in the regulation of TBK1 phosphorylation (38), CK2 does not interact with or enhance the activity of PPM1B (data not shown). Together, our results suggest a scenario in which CK2 may regulate IRF3 phosphorylation through two independent mechanisms, as follows: on one hand, CK2 inhibits IRF3 phosphorylation through its bona fide phosphatase PP2A; in contrast, CK2 controls IRF3 phosphorylation by tempering TBK1 activation, through an unidentified intermediate molecule.

Although it is beneficial for host to prevent unwanted or prolonged IFN response, viral tactics of dampening IFN response can cripple host defense mechanisms, leading to exacerbated or chronic infections. In this regard, finding strategies to overcome viral immune evasion/interference mechanisms is highly desirable. Our study demonstrated that knocking down CK2 expression or blocking CK2 activity appears to be sufficient to activate TBK1 and elicit low level of IFN response at steady state. This phenomenon suggests an appealing scenario in which TBK1 could be activated without engaging any upstream innate receptors. Conceivably, this offers an opportunity to prevent or treat infection by viruses that are capable of subverting IFN-inducing innate receptor signaling. As a proof of concept, blocking CK2 activity by TBB was indeed able to create an antiviral state, alleviating viral infection and replication (Figs. 7, 8). It will be tempting to test whether inducing optimal IFN response was indeed able to create an antiviral state, alleviating viral infection and replication (Figs. 7, 8). It will be tempting to test whether inducing optimal IFN response was indeed able to create an antiviral state, alleviating viral infection and replication (Figs. 7, 8).

In summary, we report in this work that CK2 is critically involved in the regulation of TBK1 and IRF3 activation in antiviral IFN response. This study not only unravels a new mechanism underlying innate receptor-triggered IFN response and host defense, but also suggests a viable approach to overcome viral immune evasion/interference mechanisms for the treatment of debilitating viral infections.

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Disclosures

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References


