Negative Regulation of Virus-triggered IFN-β Signaling Pathway by Alternative Splicing of TBK1*

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Induction of Type I IFNs is a central event in antiviral responses and must be tightly controlled. The protein kinase TBK1 is critically involved in virus-triggered type I IFN signaling. In this study, we identify an alternatively spliced isoform of TBK1, termed TBK1s, which lacks exons 3–6. Upon Sendai virus (SeV) infection, TBK1s is induced in both human and mouse cells and binds to RIG-I, disrupting the interaction between RIG-I and VISA. Consistent with that result, overexpression of TBK1s inhibits IRF3 nuclear translocation and leads to a shutdown of SeV-triggered IFN-β production. Taken together, our data indicate that TBK1s plays an inhibitory role in virus-triggered IFN-β-signaling pathways.

Innate immunity is the first line of defense against viral and microbial pathogens. There are at least two pattern-recognition receptors (PRRs) that detect the presence of viral double-stranded RNA (dsRNA)1,2 (1,2): a subfamily of TLRs (TLR3, -7, -8, -9) (3–6); and retinoic acid-inducible gene I-like helicases (RLHs), which include RIG-I and melanoma differentiation-associated gene 5 (MDA5)(7, 8). TLR3, which detects extracellular viral dsRNA internalized into endosomes, recruits a TIR domain-containing adaptor-inducing IFN-β (TRIF) to the receptor upon ligand stimulation (9–12). RIG-I and MDA-5 are cytoplasmic sensors of virally derived dsRNA sharing caspase recruitment domains (CARDs) at the N terminus followed by a DexD/H-box helicase domain at the C terminus. Using knock-out mice, it has been shown that RIG-I detects various RNA viruses including Sendai virus (SeV), vesicular stomatitis virus (VSV), influenza virus, hepatitis C virus (HCV), and Japanese encephalitis virus (JEV), whereas MDA5 recognizes poly(I:C) and is essential for triggering the host response to the picornavirus encephalomyocarditis virus (EMCV)(7, 8). Both RIG-I and MDA5 interact with viral dsRNA in the cytosol via the helicase domain and initiate downstream signaling cascades via the CARDs by association with virus-induced signaling adaptor (VISA, also known as MAVS, IPS-1, or CARDIF) (13–16). Engagement of any of these receptors triggers rapid production of type I interferon (IFN-α/β) and consequently establishes the innate immune status against virus replication (1, 2).

TANK binding kinase 1 (TBK1) (17–19), which was originally identified in the context of regulation of NF-κB activity, is activated downstream of TRIF and VISA in response to viral dsRNA. TBK1 can phosphorylate IFN-β-regulatory factor (IRF)-3 and IRF-7 in vitro (20–22). The phosphorylated IRF3 and IRF7 in turn form homodimers or heterodimers, translocate into the nucleus and induce the expression of type I IFN as well as IFN-inducible gene. Knockdown assays and gene-targeting studies have shown that TBK1 is essential for type I interferon production in TLR3 and RLH signaling pathway (22–25).

Although it is essential for provoking innate immune response and enhancing adaptive immunity against virus, host antiviral response must be tightly controlled to prevent harmful effects resulting from excessive activation. In this study, we identify an alternatively spliced isoform of TBK1, termed TBK1s, which lacks exons 3–6, and negatively regulates virus-triggered IFN-β-signaling pathway.

EXPERIMENTAL PROCEDURES

cDNA Constructs and Reagents—Mouse TBK1 and mouse TBK1s sequences were amplified by PCR using cDNA from SeV-infected bone marrow-derived dendritic cells (BM-DCs) and confirmed by sequencing. PCR primers were based on

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4 The abbreviations used are: dsRNA, double-stranded RNA; RIG-I, retinoic acid-inducible gene I; MDA5, melanoma differentiation-associated gene 5; TRIF, TIR domain-containing adaptor-inducing IFN-β; VISA, virus-induced signaling adaptor; TBK1, TANK-binding kinase 1; IRF3, interferon regulatory factor 3; SeV, Sendai virus; CARD, caspase recruitment domain; IFN, interferon; DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; BM-DC, bone marrow-derived dendritic cells.
the NCBI GenBank™ sequence with accession number NM_019786. The open reading frame of TBK1 and TBK1s was cloned into pcDNA3.1-HA vector (Invitrogen) at BamHI/EcoRI sites and BamHI/Xhol sites, respectively. IRF3 was amplified by PCR with NCBI GenBank™ accession number NM_016849 and cloned into pEGFP-N1 vector (Invitrogen), 100 units/ml penicillin, and 100 units/ml streptomycin. The method for generation of bone marrow–derived macrophages was described previously (26). Transient transfection of HEK293T cells was done with the same volume medium containing 20% fetal calf serum. After infection, cells were collected and used for some experiments.

Immunoprecipitation and Immunoblot Analysis—Cultures of HEK293T cells in 6-well plates were transfected with various combinations of plasmids. Immunoprecipitation and immunoblot analysis were performed as described before (27).

Expression of the VISA coding sequence was amplified by PCR with NCBI GenBank™ accession number NM_016849 and cloned into pcDNA3.1-HA vector at BamHI/EcoRI sites. cDNA encoding amino acids 1–133, 133–423, and 423–501 of VISA was amplified by PCR and subcloned into pcDNA3.1-HA vector at BamHI/Xhol sites. The expression construct of the pLuc-IFN-β was a generous gift of Katherine A. Fitzgerald (The University of Massachusetts Medical School, Worcester, MA). The FLAG-tagged RIG-I plasmid was obtained from Takashi Fujita (Institute for Virus Research, Kyoto University, Japan). cDNA encoding amino acids 1–218 and 219–925 of RIG-I was amplified by PCR and cloned into pcDNA3.0-FLAG vector (Invitrogen) at EcoRI/XhoI sites. HA-tagged VISA and Myc-tagged TBK1s were provided by Hongbing Shu (Wuhan University, Wuhan, China). GST-RIG-I tag and VISA from Chen Wang (Shanghai Institutes for Biological Sciences, Shanghai, China). The pISRE-Luc was from Stratagene. All reagents were obtained from Sigma unless otherwise stated.

Reverse Transcription and Quantitative Real-time PCR—Total RNA was extracted from cultured cells with TRIzol (Invitrogen), and cDNA was prepared as described previously (28). Quantitative real-time PCR (Q-PCR) analysis was performed using the ABI PRISM® 7900HT (ABI). The primers used for PCR are listed in Table 1. Data were normalized according to the level of GAPDH expression in each sample. The primers were used for PCR are listed in Table 1. Data were normalized according to the level of GAPDH expression in each sample.

Nuclear Extracts—Nuclear extracts were prepared as previously described (28).

Immunofluorescence Microscopy—Cells were fixed with 4% formaldehyde, permeabilized in 0.5% Triton X-100, blocked, and nuclei were stained using 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured with a Leica TCS SP2 laser confocal microscope.

Reporter Assays—Cells (1 × 10^6/well in 24-well plates) were transfected with the indicate amount of expression plasmids combined with 100 ng of indicated reporter genes and 40 ng of pRl-TK (Clontech). The total DNA concentration was kept constant by supplementing with empty vector pcDNA3.0. In some experiments, cells were infected with SeV after transfection. In all experiments, cells were lysed, and reporter activity was analyzed with the Dual-Luciferase Reporter Assay System (Promega). The values represented the average of three independent experiments with variability shown by the error bars.

Statistics—All measurements were performed in at least three independent experiments, and the means ± S.D. were calculated. The Student’s t test was used to compare two independent groups. For all tests, values of p < 0.05 were considered statistically significant.

### Table 1: Sequences of PCR primers

| Gene     | Sense          | Antisense       |
|----------|----------------|-----------------|
| Mouse TbK1 | 5′-ATGCAGACCCCACTCCAAATCT-3′ | 5′-CTTACCACGGGCTAATCTCCA-3′ |
| Mouse Ifnb | 5′-CGGCTTCTCCTGCTCT-3′ | 5′-CTTCACGGTCGTTGAGTCGA-3′ |
| Mouse Ip-10 | 5′-TTGCTCCTGTCCTGCTGTA-3′ | 5′-CTTACGCGGCTGATCACTTCA-3′ |
| Human Ifnb | 5′-TGCAGATCGAGGCTTCCTG-3′ | 5′-AGGTGTAACACTGACAAC-3′ |
| Human Ip-10 | 5′-TGGCATACAGGAAGCTTCT-3′ | 5′-GGATGTTGTTGAGTCGAAC-3′ |
| Human Il-8 | 5′-ATGGGGTCCATGAAAACTGAC-3′ | 5′-GGTTGCGGAGGTGCTGATGA-3′ |

### Gene Expression Analysis

- **Mouse Tbk1**: RT-qPCR was performed to analyze the expression levels of Tbk1 in various cell lines. The results showed statistically significant differences among the groups.

- **Human Tbk1**: Quantitative real-time PCR (Q-PCR) analysis was performed to measure the expression levels of Tbk1 in different conditions. The results indicated a significant increase in expression under specific conditions.

- **Human Il-8**: The expression of Il-8 was assessed by RT-qPCR in various cell lines. The analysis revealed a significant upregulation in treated samples compared to controls.

### Discussion

The data presented in this study highlight the regulatory role of TBK1s in RIG-I signaling pathway. The results suggest that TBK1s may serve as a key mediator in the induction of antiviral responses, possibly by modulating the expression of key genes such as Tbk1.

The findings support the hypothesis that TBK1s play a crucial role in the host defense against viral infections, and provide new insights into the mechanisms underlying the antiviral response.

In conclusion, the investigation of TBK1s in the RIG-I signaling pathway offers potential therapeutic targets for the treatment of viral infections.
RESULTS

Identification of a Splice Variant of TBK1, termed TBK1s, Which Lacks Exons 3–6

The mouse Tbk1 gene is organized into 21 exons and 20 introns (Fig. 1A). Exons 2–6 (corresponding to amino acids 1–234) encode the S_TKc domain, which mediates the phosphorylation of IRF3 and IRF7. RT-PCR for TBK1 on RNA isolated from BM-DC infected with 10 HAU/ml Sendai virus reveals two cDNA species of 2190 and 1567 bps, respectively (Fig. 1B). Sequence analysis shows that the larger band is identical to the published sequence of full-length Tbk1, while the smaller band represents an alternative splicing isoform of Tbk1s, lacking exons 3–6 and subsequently referred to as Tbk1s. Excision of exons 3–6 results in translation from the second ATG and leads to an in-frame deletion of the kinase domain (amino acids 1–234) (Fig. 1A). Moreover, analysis of genomic locus that contains the human TBK1 sequence suggests a similar splice acceptor-donor site that could lead to a splice variant similar to the mouse TBK1s isoform. To further investigate the existence of the Tbk1 spliced isoform in human, we designed human Tbk1 isform-specific primers and Tbk1s isform-specific primers based on the difference of splicing site (human Tbk1 NCBI GenBank™ Accession Number: NM_013254). Human hepatoma Huh7 cells were infected with Sendai virus and the expression of human TBK1 and TBK1s mRNA were measured by RT-PCR using human Tbk1 isform-specific primers and human Tbk1s isform-specific primers, respectively. As shown in Fig. 1C, a Tbk1s-specific PCR product (~60 bp) was detected in cells infected with SeV. To further confirm the existence of the TBK1 spliced isoform, we analyzed TBK1s protein expression. To assess this, we used rabbit polyclonal antibodies against amino acids 355–729 of mouse TBK1 (Santa Cruz Biotechnology) to detect the presence of TBK1s protein, and cell lysates from 293T cells transfected with TBK1s plasmid served as positive control. In addition to an endogenous ~75-kDa band (TBK1 protein), an endogenous ~55-kDa protein (TBK1s protein), which was consistent with exogenous expression of TBK1s in 293T was recognized in BM-DC infected with SeV (Fig. 1D). Furthermore, TBK1s protein could be induced in Huh7 cells treated with SeV or IFN-γ (Fig. 1E).

Compared with the constitutive expression of TBK1, the expression of TBK1s was inducible and lower. The tissue distribution of TBK1s expression, assessed by immunoblot analysis, showed that brain, heart, kidney, lymph node, and thymus had relative high expression of TBK1s protein (Fig. 1F). Intriguingly, TBK1s protein was more abundant than TBK1 protein in heart and kidney. Our data not only demonstrated the existence of the Tbk1 spliced isoform in both mouse and human cells, but also led us to further investigate the function of TBK1s.

The Kinase Domain of TBK1 Is Critical for the Induction of IFN-β

Initial experiments using MEF cells reconstituted with plasmids encoding TBK1 or TBK1s, and cells were infected with SeV after
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It has been reported that TBK1 is essential for Type I interferon production in virus-triggered signaling pathway. Because TBK1s is an alternative splicing isoform of TBK1, which lacks the kinase domain, we hypothesized that TBK1s could play an inhibitory role on this pathway. To test this hypothesis, 293T cells were transfected with TBK1s plasmid or the vector control, and infected with different doses of SeV after 24 h of transfection. Reporter gene assays, TBK1s inhibited SeV-triggered activation of ISRE and IFN-β (Fig. 3A). Furthermore, we measured the effect of TBK1s on the induction of type I IFN-inducible genes including Ifnb, IP-10, and IL-8 genes. As shown in Fig. 3B, TBK1s inhibited expression of Ifnb and IP-10 mRNA induced by SeV, while TBK1s had no effect on expression of IL-8 mRNA. These data suggest that TBK1s specifically inhibits SeV-induced IFN-β activation. We also assessed the expression of transfected TBK1s protein. Immunoblot analysis with anti-TBK1 antibody showed that ectopic expression of TBK1s expressed once higher than that of endogenous TBK1 protein (Fig. 3C). To further evaluate the effect of TBK1s on the activation and nuclear translocation of IRF3, nuclear extracts from SeV-infected 293T cells were analyzed for the presence of IRF3. The accumulation of IRF3 in the nucleus triggered by SeV was inhibited when 293T cells were transfected with TBK1s plasmid, whereas NF-κB subunit p65 translocation was not affected (Fig. 3D). Together, these observations show that TBK1s nega-
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**FIGURE 4.** TBK1s inhibits RIG-I but not VISA- or TBK1-mediated activation of IFN-β promoter. A–C, IFN-β promoter reporter was transfected into 293T cells together with pRL-TK, increasing concentrations of TBK1s (100 ng, 200 ng, 400 ng), and (A) TBK1 (100 ng); (B) RIG-I (200 ng), or (C) VISA (100 ng). After 24 h of transfection, cells were subjected to the dual-luciferase assay (above) and immunoblot assays with anti-TBK1 antibody (below) or the indicated antibody. Error bars in A–C indicate ± S.D. between duplicates. The ratios of TBK1-TBK1s are shown as indicated.

**TABLE 1.** The negative effect of TBK1s on SeV-triggered IFN-β promoter analysis with anti-TBK1 antibody showed that TBK1s could inhibit the SeV-induced IFN-β promoter activation of the IFN-β promoter compared with wild-type TBK1s. Consistent with these data, the TBK1s mutant 1–371 was able to effectively inhibit SeV-induced activation of the IFN-β promoter compared with wild-type TBK1s (Fig. 5F). To illustrate the physiological role of TBK1s, we also analyzed the endogenous interaction of TBK1s with RIG-I. We performed co-immunoprecipitation experiments of lysates of L929 after SeV infection, which showed specific interaction between endogenous RIG-I and TBK1s (Fig. 5G). In contrast, the endogenous interaction between TBK1 and RIG-I could not be detected (Fig. 5G).

**DISCUSSION**

Negative control of Type I IFN production is an essential physiological process and can be achieved at multiple levels. For instance, LGP2, a RNA helicase protein, which lacks CARD domains at its N terminus, can be induced by virus and serves as a negative regulator by sequestering viral RNA from RIG-I and MDA5 (29–32). RNF125, an E3 ubiquitin ligase, negatively regulates RIG-I-induced antiviral signaling through mediation of RIG-I degradation (33). In this study, we demonstrate a new mechanism of negative regulation of Type I IFN production by alternative splicing of TBK1 pre-mRNA.

Alternative splicing is a mechanism that allows for individual genes to express multiple mRNAs that encode proteins with diverse and even antagonistic function. Indeed, alternative splicing has been described for various adaptors and transcrip-
tion factors involved in antiviral response, including IRF family members (34–39). For instance, it has been reported that IRF-5 alternatively spliced isoforms are differentially regulated by at least two alternative promoters, while the splicing of IRF-3 and IRF-3a transcripts may be regulated in a tissue-specific manner (34, 35). Here, we demonstrated that the expression of TBK1s, as compared with constitutive expression of TBK1, can be induced by SeV infection or IFN-β treatment. Furthermore, expression of Tbk1s was significantly higher in peripheral blood mononuclear cell (PBMC) from the HCV-infected patients than those in the healthy controls, while it was down-regulated in PBMC from the HCV-infected patients treated with IFN-α/ribavirin whose HCV RNA turn to negativity (HCV Neg) (supplemental Fig. S2 and Table S1). Consistent with a previous report (40), the expression of Tbk1 was not found to alter significantly upon HCV infection. Such a regulated production of TBK1s would result in the controlled inhibition of type I IFN production. Intriguingly, the ratio of TBK1s protein expression versus TBK1 protein expression in heart and kidney were really high, indicating TBK1s could be regulated in a tissue-specific manner. However, differential isoform expression and regulation of TBK1 remain to be addressed.

Because TBK1s does not contain the kinase domain but does maintain the ability to bind IRF3 (data not shown), it was proposed that the mechanism by which TBK1s inhibits virus-triggered signaling pathway probably involves sequestration of TBK1 from IRF3, thereby preventing IRF3 activation. While the present study is in agreement with the negative regulatory role

FIGURE 5. TBK1s binds to RIG-I and disrupts the interactions of RIG-I with VISA. A–E, immunoprecipitation (IP) and immunoblot (IB) analysis of lysates of HEK293T cells expressing various recombinant proteins (total amount: 2 μg), using the indicated antibodies. A, FLAG-RIG-I together with HA-TBK1 or HA-TBK1s. B, HA-TBK1 or HA-TBK1s together with vector or FLAG-RIG-I. C, schematic diagram of TBK1s truncation mutants used in this study (above). CC, coil-coiled domain. CC, coil-coiled domain. FLAG-RIG-I (WT) together with HA-TBK1s wild type or truncation mutants. D, schematic diagram of RIG-I truncation mutants used in this study (above). CARD, caspase recruitment domain. HA-TBK1s together with the indicated FLAG-RIG-I wild type or truncation mutants. Arrow indicated HA-TBK1s. E, HEK293T cells (1 × 10⁶) were transfected with an empty vector, HA-TBK1s expression vector or TBK1s mutant 1–371 (4 μg). After 24 h of transfection, cells were infected with 10 HAU/ml SeV for 4 h, F, HA-TBK1s or TBK1s mutant 1–371 was transfected into 293T cells together with pRL-TK and IFN-β promoter reporter. After 24 h of transfection, cells were infected with 10 HAU/ml SeV for 16 h and subjected to the dual-luciferase assay (above) and immunoblot assay with anti-HA antibody (below). Error bars indicate ± S.D. between duplicates. **, p < 0.01. G, L929 cells (1 × 10⁶) were infected with 10 HAU/ml SeV for the indicated time. Cell lysates were incubated with 2 μl of RIG-I antibody or control IgG for immunoprecipitation followed by immunoblotting, using the indicated antibodies. The ratio of TBK1-TBK1s was shown as indicated.
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of TBK1s, differences were found in the proposed mechanism of inhibition. Actually, TBK1s inhibits RIG-I but not TBK1-mediated activation of ISRE and the IFN-β promoter, and TBK1s negatively regulates virus-triggered IFN-β signaling pathway by disrupting the interaction of RIG-I with VISA. It is reasonable to assume that TBK1s targets RIG-I instead of TBK1. First, the expression of TBK1s is lower than that of TBK1 even upon Sendai virus infection (Figs. 1 and S5); second, IRF3 phosphorylation mediated by TBK1 is not blocked in the presence of TBK1s (data not shown).

In this study, we show that the coiled-coil domain of TBK1s is responsible for the interaction between RIG-I and TBK1s. Actually, full-length TBK1 also contains the coiled-coil domain. It is not yet clear why TBK1 does not bind to RIG-I but TBK1s does. Given the fact that kinase-defective TBK1 (K38A) can bind to RIG-I, we suggest that it would be related to the kinase activity (supplemental Fig. S2). Moreover, RIG-I binds to TBK1s via its CARDs. It has been reported the Lys-172 residue can bind to RIG-I, we suggest that it would be related to the proline-rich domain. It is not yet clear why TBK1 does not bind to RIG-I but TBK1s, differences were found in the proposed mechanism of inhibition. Actually, TBK1s inhibits RIG-I but not TBK1-mediated activation of ISRE and the IFN-β promoter, and TBK1s negatively regulates virus-triggered IFN-β signaling pathway by disrupting the interaction of RIG-I with VISA. It is reasonable to assume that TBK1s targets RIG-I instead of TBK1. First, the expression of TBK1s is lower than that of TBK1 even upon Sendai virus infection (Figs. 1 and S5); second, IRF3 phosphorylation mediated by TBK1 is not blocked in the presence of TBK1s (data not shown).

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