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TBK1-like transcript negatively regulates the production of IFN and IFN-stimulated genes through RLRs-MAVS-TBK1 pathway

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ABSTRACT

TANK-binding kinase 1 (TBK1) is an essential serine/threonine-protein kinase required for Toll-like receptor (TLR)- and retinoic acid-inducible gene I (RIG-I) -mediated induction of type I IFN and host antiviral defense. In the present study, TBK1-like transcript, namely TBK1L, was cloned from zebrafish. Compared with TBK1, TBK1L contains an incomplete S_TKc domain, and lacks UBL_TBK1_like domain. Realtime PCR showed that TBK1L was constitutively produced in embryos, early larvae and ZF4 cells, and unchanged in ZF4 cells following SVCV infection. Overexpression of TBK1 but not TBK1L resulted in significant activation of zebrafish IFN1 and IFN3 promoters. Similarly, TBK1L had little impact on the antiviral state of the cells. However, the overexpression of TBK1L negatively regulated the induction of zebrafish IFN1 and/or IFN3 promoters mediated by the retinoic acid-inducible gene I-like receptors (RLRs), MAVS and TBK1. In addition, the overexpression of TBK1L in zebrafish embryos led to the decreased production of many IFN-stimulated genes induced by TBK1. Collectively, these data support that zebrafish TBK1L negatively regulates RLRs-MAVS-TBK1 pathway.

1. Introduction

The innate immune system senses invading pathogens through evolutionarily conserved pattern recognition receptors (PRRs). At least four major classes of PRRs have been identified in vertebrate, including Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-oligomerization domain (NOD)-like receptors (NLRs), peptidoglycan recognition proteins (PGRPs) [1,2]. In response to virus infection, TLRs, RLRs and NLRs could detect viral pathogen-associated molecular patterns (PAMPs), and activate downstream signaling transduction to initiate the transcription of IFN and IFN-stimulated genes (ISGs) [3]. Among these PRR members, many cytosolic receptors such as RIG-I, MDA5, NOD2 recruit mitochondria antiviral-signaling protein (MAVS) to activate TBK1 [4]. Activated TBK1 then phosphorylates IRF3 and IRF7, which in turn initiate the expression of type I IFNs. Secreted IFNs further activate the tyrosine residues of the Janus kinase (JAK) and signal transducers and activators of transcription (STAT) proteins, and initiate antiviral related genes expression [5].

As a critical kinase involved in antiviral immunity, inflammatory responses [6] and the regulation of autophagy [7–9], the activity of TBK1 must be tightly controlled to maintain immune homeostasis. TBK1 activity can be regulated by multiple molecules in a variety of ways. Dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2), Glycogen synthase kinase-3 beta (GSK3β), Src homology 2 domain–containing inositol-5-phosphatase 1 (SHIP1), Protein Phosphatase, Mg^{2+}/Mn^{2+} Dependent, 1B (PPM1B) and glucocorticoids modulate TBK1 activity targeting its phosphorlation [10–14]. Suppressor of cytokine signaling 3 (SOCS3), mindbomb E3 ubiquitin protein ligase 2 (MIB2), E3 ubiquitin ligase (Nrdp1) and Tripartite Motif Containing 27 (TRIM27) modulate TBK1 activity targeting its ubiquitination [15–18]. IFN-stimulated gene 56 (ISG56) and suppressor of IκB kinase epsilon (SIKE) also mediate negative-feedback regulation of virus-triggered induction of type I

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IFNs and cellular antiviral responses by suppressing TBK1-containing complex formation [19]. However, whether TBK1 isoforms or TBK1-like transcripts contribute to the modulation of TBK1 activity still need to be fully elucidated.

In mouse, an alternatively spliced isoform of TBK1, termed TBK1s, was identified. This isoform can negatively regulate virus-triggered IFN-β signaling pathways by targeting RIG-I rather than TBK1 [20]. In the present study, we identify the TBK1-like transcript, termed TBK1L, which negatively regulates the production of IFNs and ISGs induced by RLRs-MAVS-TBK1 pathway. Our findings reveal a new negative regulatory mechanism for TBK1 activity and type I IFN production in antiviral innate immune response.

2. Materials and methods

2.1. Cells and virus

Zebrafish embryonic fibroblast cell line (ZF4) were cultured in DMEM/F-12 medium (ThermoFisher), and Epithelioma papulosum cyprini (EPC) in Medium 199 (ThermoFisher) at 28 °C. Spring viremia of carp virus (SVCV) was propagated in EPC cells at 25 °C.

2.2. Plasmids

Based on zebrafish mRNA sequences (GenBank accession No: NM_001044748), the ORFs of TBK1 and TBK1L were amplified with primer pairs p3 × FLAG-TBK1F/p3 × FLAG-TBK1R, and inserted into p3 × FLAG-CMV-14 (Siga-Aldrich) vector. The ORF of RIG-Ib (RIG-I normal form) were amplified with primer pairs pcDNA3.1RIG-IF/pcDNA3.1RIG-IR, and inserted into pcDNA3.1 (Invitrogen) vectors. The primer sequences used for plasmid construction were listed in Table 1 or described in our previous study for pcDNA3.1-MDA5a plasmid (MDA5 normal form) [21].

2.3. Gene expression patterns in developmental stages and ZF4 cells following SVCV infection

For examining the expression of development stages, 5 samples including 6, 24, 48, 72 and 120 h post-fertilization (hpf) were used for RNA extraction. For SVCV infection, ZF4 cells were passaged in six-well plates at 1×10^6 cells per well, and 24 h later, these cells were infected with SVCV at a multiplicity of infection (MOI) of 1, and then collected at 6, 24, 48 and 72 h post-infection (hpi) for RNA extraction. The isolated RNA was firstly treated with RNase-free DNase I (Fermentas Life Sciences, Vilnius, Lithuania), and then incorporated into first strand cDNA by Superscript reverse transcriptase (Fermentas Life Sciences) and oligo (dT) primer. Quantitative real time PCR was performed on a BIO-RAD CFX96 Real-Time System under the following conditions: 3 min at 95 °C, followed by 45 cycles of 15 s at 94 °C, 15 s at 54 °C and 30 s at 72 °C. The relative expression of target gene was normalized to the expression of GAPDH, and expressed as arbitrary units. A probability level of $P < 0.05$ was considered statistically significant using the paired student's *t*-test. The primer sequences used for Real time PCR are listed in Table 1.

2.4. Antiviral assay

EPC cells seeded in 24-well plates at a concentration of 3×10^5 cells per well were transfected with 500 ng p3 × FLAG-CMV-14 vector, TBK1-flag, TBK1L-flag, respectively. Twenty-four hours post-transfection, transfected cells were washed and infected with SVCV at an MOI of 5. At 48 h post-infection, the culture supernatants were collected for the determination of virus titres by standard plaque assay. Cell monolayers were then fixed in 10% paraformaldehyde for 1 h before being stained with 0.5% crystal violet for the observation of cytopathic effect.

2.5. Luciferase activity assay

For luciferase activity assay, EPC cells seeded overnight in

Table 1
Primer sequences used in this study.

Primer	Sequence (5′ to 3′)	Application
p3 × FLAG-TBK1F	GTCAAGCTTTCTACAGTCATCATGCAGAGT	TBK1-flag
p3 × FLAG-TBK1R	GAAGGTACCATCCGCTCCACTGTCCTCA	TBK1L-flag
pcDNA3.1RIG-IF	CGCGGATCCACCATGGCGTACGAGCTGGAGAAGGAGA	pcDNA3.1RIG-I
pcDNA3.1RIG-IR	CGGGGTACCGTTGACCAGCGCCCATG	
TBK1LF	ATGATGTGTCACGGAGCCGCACATGGA	Realtime PCR
TBK1LR	CACCTCGTTCAGGATCGCGTAGAGACT	
IFN1F	GTCAGGACTAAAAACTTCAC	
IFN1R	TCTTAATACACGCAAAGATGAGAACT	
IFN2F	CCTCTTTGCCAACGACAGTT	
IFN2R	CGGTTCCTTGAGCTCTCATC	
IFN3F	TTCTGCTTTGTGCAGGTTTG	
IFN3R	GGTATAGAAACGCGGTCGTC	
mxaF	GGAGAATCAGTTACAAAACCT	
mxaR	GATTGTCTCTTGCCTTGTAACA	
mxbF	AATGGTGATCCGCTATCTGC	
mxbR	TCTGGCGGCTCAGTAAGTTT	
mxcF	GAGGCTTCACTTGGCAACTC	
mxcR	TTGTTCCAATAAGGCCAAGC	
mxeF	TGAAGATGGCATCCACAGTT	
mxeR	TCTTTCTGCAAGCAGGGGT	
PKZF	GGAGCACCGTACAGGACATT	
PKZR	CTCGGGCTTTATTTGCTCTG	
RSAD2F	AGCAGATCACCGCTCTCAAT	
RSAD2R	CCAGACACTGGATGCTCTGA	
GAPDHF	GTAACTCCGCAGAAAAGCCAGAC	
GAPDHR	CAAAAGAAACTAACACACACACA	
SVCV-GF	GCCGATTATCCTTCCACCTT	
SVCV-GR	TCACTTGCCCTTCCCACTCT	