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Functional characterization of duck TBK1 in IFN-β induction

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ABSTRACT

TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) serves as hub molecule at the crossroad of multiple signaling pathways of type I interferon (IFN) induction. The importance of TBK1 in innate immunity has been demonstrated in mammalian, however the characterization and function of TBK1 in avian remains largely unknown. In this study, we cloned duck TBK1 (duTBK1) from duck embryo fibroblasts (DEFs) for the first time, which encoded 729 amino acids and had a high amino acid identity with goose and cormorant TBK1s. The duTBK1 showed a diffuse cytoplasmic localization in DEFs and was extensively expressed in all tested tissues. Overexpression of duTBK1 induced IFN-β production through the activation of IRF1 and NF-κB in DEFs. The N-terminal kinase domain and the ubiquitin-like domain in middle of duTBK1 played pivotal roles in IFN-β induction as well as in IRF1 and NF-κB activation. Furthermore, knockdown of duTBK1 by small interfering RNA significantly decreased poly(I:C)- or Sendai virus (SeV)-induced IFN-β expression. In addition, duTBK1 expression dramatically reduced the replication of both duck reovirus (DRV) and duck Tembusu virus (DTMUV) in DEFs. These results suggested that the duTBK1 played a pivotal role in mediating duck antiviral innate immunity.

1. Introduction

The trigger of antiviral innate immune response relies on the recognition of pathogen-associated molecular patterns (PAMPs) derived from viruses by specialized receptors called molecular pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) [1,2]. PRRs-induced signal transduction pathways are activated by detecting the PAMPs, including viral DNA or RNA, transcription products, and replicative intermediates [3]. Upon recognition of PAMPs by plasma membrane-localized or cytosolic PRRs, different kinds of adaptor proteins, such as TRIF, MAVS, MyD88, and STING, are recruited by different PRRs to initiate the downstream induction of type I IFN and inflammatory cytokines via activation of NF-κB, IRF3 and AP-1, mediating the effective and further elimination of invading viruses [4–6].

TBK1, also known as NF-κB-activating kinase (NAK) or TRAF2-associated kinase (T2K), is a member of non-canonical IκB kinase (IKK) family and can directly phosphorylate IKKβ, thus activating NF-κB through inducing the degradation of IκB in human, mouse [7,8]. Furthermore, TBK1 has also been reported to assemble with TRAF3 and TANK, upstream adaptor proteins of TBK1, to phosphorylate IRF3, leading to the nucleus translocation of IRF3 and inducing the expression of type I IFN [9,10]. Mammalian TBK1 has been described as a protein of 729 amino acids consisting of a kinase domain (KD) at N-terminal, a middle ubiquitin-like domain (ULD) which controls the activity of kinase domain, and a coiled coil-containing domain (CC) in the C-terminal of unknown function [11,12]. The KD and ULD domains are both essential for the phosphorylation of downstream IRF3 and NF-κB, thus triggering the IFN production [13,14].

Although the characterization and function of TBK1 in antiviral immunity has been identified in mammals, the characterization of TBK1 gene and its roles in avian innate immunity remain largely unknown. Recently, chicken TBK1 has been reported to contribute to IFNβ induction against chicken avian leukosis virus subgroup J (ALV-J) infection [15]. In the present study, a full-length duck TBK1 (duTBK1) gene was characterized for the first time. Moreover, we revealed that the duTBK1 played an important role in inducing duck IFN-β expression and possessed the ability to dramatically inhibit proliferation of both ducks reovirus (DRV) and duck Tembusu virus (DTMUV).

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2. Materials and methods

2.1. Cells, tissues, viruses, and reagents

Duck embryo fibroblast cell line was obtained from ATCC and cultured in Minimum Essential Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco). Tissues for TBK1 expression analysis obtained from 1-month-old healthy cherry ducks, including lung, liver, spleen, heart, kidney, cerebrum, thymus, duodenum, caecum and bursa of Fabricius were snap-frozen into liquid nitrogen and stored at −80 °C for further analysis. The NF-κB specific inhibitor BAY11-7082 was purchased from Selleck (USA). Sendai virus (SeV) was obtained from the Center of Virus Resource and Information, Wuhan Institute of Virology, Chinese Academy of Sciences. Duck reovirus (DRV) strain JZ061214 (GenBank Accession Number: JX852433) was a gift of Dr. Xueying Hu (College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, China). DTMUV strain MC was a clinically-isolated pathogenic strain that has been sequenced (GenBank Accession Number: KX452096). Poly(I:C) was purchased from Sigma (St Louis, MO, USA). Phospho-TBK1 (Ser172) monoclonal antibody (#5483) was purchased from CST (USA) and TBK1 polyclonal antibody (A2573) was obtained from ABclonal (China).

2.2. Cloning and sequence analysis of duTBK1

According to the predicted duTBK1 coding sequence (GenBank accession number XM_005029450), the duTBK1-5′GSP and duTBK1-3′GSP primers (Table 1) were designed to clone 5′- and 3′- terminals region of duTBK1 with SMARTer RACE 5′/3′ Kit (Takara). DNA fragments were cloned into pRACE vector for sequencing. After analyzing the coding sequence of duTBK1, a pair of primers (duTBK1-CDS-F and duTBK1-CDS-R, Table 1) was designed to amplify the coding sequence of duTBK1. All the cDNAs used above were amplified by RT-PCR with total RNA extracted from DEFs.

The full-length mRNA sequence of duTBK1 has been uploaded to GeneBank (accession number MG772817). A multiple sequence alignment was performed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). MEGA4 was used to construct a phylogenetic tree. The functional domains of duTBK1 were predicted with the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/).

Table 1
PCR primers used in this study.

Primer	Sequence (5′-3′)
duTBK1-3′GSP	GATTACGCCAAGCTTGAGCGAGCAGTTTTGAGGAAGGAGCATC
duTBK1-5′GSP	GATTACGCCAAGCTTCAGGCATCTCCCAGCTCCACTCAATTGG
duTBK1-CDS-F	GGCGAGCTCATGCAGAGCACTTCAAATTACCTCT
duTBK1-CDS-R	AATCTCGAGCTAGATGCAGTCCACATTCCGCAGG
duTBK1-KD-F	GACGAGCTCATGCAGAGCACTTCAAATTAC
duTBK1-KD-R	GAGCTCGAGCTAGAAGAACTGGTCAAATCC
duTBK1-ULD-F	AAAGAGCTCATGGCAGAGACAAGTGACATC
duTBK1-ULD-R	GAGCTCGAGCTACACTACGAAGATAGGATTCT
duTBK1-CC-F	AAAGAGCTCATGAGCAGGGAGCCTGTGAAC
duTBK1-CC-R	GAGCTCGAGCTAGATGCAGTCCACATTC
duIRF1-DN-F	AAAGAGCTCACAAAGGATCAGAAGAAAGAAA
duIRF1-DN-R	GAGCTCGAGTTACAAGCCACAGGAGATG
qTBK1-F	TGATCTATGAAGGTCGGCGTTT
qTBK1-R	CTGCTCACTACGAAGATAGGATTCTC
qβ-actin-F	GGCCAGGTCATCACCATTG
qβ-actin-R	GATGCCACAGGACTCCATACC

2.3. Distribution analysis of duTBK1 in different tissues

Total cellular RNA from different tissues of healthy cherry ducks was extracted with Trizol reagent (Invitrogen) and then reverse transcribed to cDNA with iScript cDNA Synthesis Kit (Bio-Rad). The cDNA was used to perform quantitative real-time PCR (qRT-PCR) with a pair of specific primer (qTBK1-F and qTBK1-R, Table 1). The SYBR green PCR assay (Applied Biosystems) was used to measure the expression of duTBK1 as follow: initial denaturation at 95 °C for 30 s followed by 40 cycles of denaturation at 95 °C for 5 s, annealing and elongation at 60 °C for 30 s. Data collection was enabled at the annealing and extension step. The melt curve protocol followed with a heating rate of 0.5 °C/2s between 65 °C and 95 °C. All samples were tested three times, and β-actin expression level was used as normalization.

2.4. Plasmid construction, transfection, and luciferase reporter assays

The luciferase reporter plasmids for IFN-β (IFN-β-Luc), NF-κB (4 × PRDII-Luc), and IRF1 (4 × PRDIII/I-Luc) have been described previously [16]. The whole coding sequence of duTBK1 was cloned into pCAGGS-Flag to generate a eukaryotic expression construct pCAGGS-duTBK1 encoding full-length duTBK1. Four truncated mutants, duTBK1-KD (aa1-299), duTBK1-ΔCC (aa1-382), duTBK1-ΔKD (aa300-729) and duTBK1-CC (aa383-729) were sub-cloned into pCAGGS-Flag using the primers listed in Table 1.

DEFs were seeded into 48 well plates. When the cells were approximately 80% confluent, they were co-transfected with 200 ng/well of expression plasmids or an empty vector and 50 ng/well of reporter plasmids (NF-κB-Luc, IFN-β-Luc, or IRF1- Luc) along with 50 ng/well of pRL-TK (Promega) by lipofectamine 2000 (Invitrogen). Cells were lysed at the indicated time and the relative firefly and renilla luciferase activity were detected with a dual-luciferase reporter assay system (Promega, USA).

2.5. Indirect immunofluorescence analysis

DEFs were seeded on 13-mm sterilized coverslips and cultured overnight to approximately 60% confluence before transfected with pCAGGS-duTBK1 or empty vector. 24 h after transfection, cells were fixed and permeabilized, blocked and subsequently incubated with antibody and 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) as previous described [17]. Finally, coverslips were imaged by a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Zena, Germany).

2.6. Western blot analysis

DEFs were cultured in 60-mm dishes to 80% confluence. Then, cells were infected by DRV or DTMUV, or transfected with indicated expression plasmids using lipofectamine 2000 reagent (Invitrogen). Cells were lysed at the indicated time with 200 μL lysis buffer (65 mM Tris–HCl, 4% sodium dodecyl sulfate, 3% DL-dithiothreitol, and 40% glycerol), followed by adding 50 μL sample buffer [2% SDS, 60 mM Tris–HCl (pH 6.8), 10% glycerol, 0.001% bromophenol blue, and 0.33% β-mercaptoethanol]. Western blot analysis was performed as previous described [17].

2.7. Knockdown of duTBK1 or duck IRF1 (duIRF1) by siRNAs

Three pairs of small interfering RNA (siRNA) sequences targeting duTBK1 and duIRF1 mRNA, respectively, were purchased from GenePharma (Shanghai, China), and the sequences are listed in Table 2. SiRNAs were transfected into DEFs by lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Subsequently, the cells were collected to perform qRT-PCR, luciferase reporter assays, or the measure of virus titers at indicated time points.

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