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TAK1 of black carp positively regulates IRF7-mediated antiviral signaling in innate immune activation

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ABSTRACT

Transforming growth factor β-activated kinase 1 (TAK1) plays a vital role in IL-1-mediated NF-κB, JNK, and p38 activation in human and mammals. However, the function of TAK1 in teleost fish still remains largely unknown. To explore the role of TAK1 during the antiviral innate immune response of teleost fish, TAK1 of black carp (*Mylopharyngodon piceus*) was cloned and characterized in this paper. The open reading frame (ORF) of black carp TAK1 (bcTAK1) consists of 1626 nucleotides and the predicted bcTAK1 protein contains 541 amino acids, which includes a N-terminal Serine/Threonine protein kinases (S/TKc) and a C-terminal coiled-coil region. bcTAK1 migrated around 75 kDa in immunoblotting assay and was identified as a cytosolic protein by immunofluorescence staining. bcTAK1 transcription in *Mylopharyngodon piceus* kidney (MPK) cells varied in response to the stimulation of poly (I:C), LPS, grass carp reovirus (GCRV), and spring viremia of carp virus (SVCV). bcTAK1 showed deficient IFN-inducing ability in reporter assay and feeble antiviral activity against GCRV and SVCV in plaque assay. However, when co-expressed with bcIRF7 in EPC cells, bcTAK1 obviously enhanced bcIRF7-mediated IFN promoter induction in reporter assay. Accordingly, the data of plaque assay demonstrated that the antiviral activity of bcIRF7 against both GCRV and SVCV was unregulated by bcTAK1. Thus, the data generated in this study support the conclusion that bcTAK1 up-regulates bcIRF7-mediated antiviral signaling during host innate immune activation, which is reported for the first time in vertebrates.

1. Introduction

Teleost fishes possess both innate immune system and adaptive immune system, depended on which these animals to protect themselves from the invasion of pathogenic microbes, such as virus [1,2]. The adaptive immune system of teleost is much simpler than that of mammals, however, the innate immune system has been well developed in teleost, which suggests the conservation of innate immunity from teleost to mammals [2,3]. Teleost utilize pathogen recognition receptors (PRRs) to detect conserved pathogen-associated molecular patterns (PAMPs) on arrange of microbes, which include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) etc. [4,5]. Teleost PRRs activate and translocate transcription activators nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and interferon regulatory factor 3/interferon regulatory factor 7 (IRF3/7) through downstream signaling post detecting PAMPs and subsequently initiate the production of pro-inflammatory cytokines and interferons (IFNs), which trigger host innate immune activation finally [6,7].

Transforming growth factor β-activated kinase 1 (TAK1, also known as MAP3K7), a member of the mitogen-activated protein kinase kinase (MAP3K) family, was first identified in the TGF-β signaling pathway in 1995 [8]. TAK1 plays diverse roles in development and immunity, such as the regulation of innate and adaptive immune response, neural fold morphogenesis, vascular development, and tumorigenesis [9,10]. TAK1 carries out these diverse biological roles through a number of signaling pathways, such as the TGF-β/BMP, Wnt/Fz, JNK, and NF-κB pathways [11]. One important and extensively studied function of TAK1 is its regulation of pro-inflammatory and innate immune signaling pathways, such as the TNF receptor, IL-1R, and TLR pathways. TAK1 stimulates the kinase activity of the IκB kinase (IKK) complex and triggers the activation of NF-κB, allowing its movement into the nucleus and the expression of pro-inflammatory cytokines [12,13]. In mammalian tissues, TAK1 is essential for responses to a variety of inflammatory

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ligands. In *Drosophila* innate immune responses, it is required for the expression of a number of antimicrobial peptides [11,14].

Compared with its mammalian counterpart, teleost TAK1 remains largely unknown. Grass carp (*Ctenopharyngodon idella*) TAK1 was reported for the first time to play essential roles in host innate immune defense against *Ichthyophthirius multifiliis* [15]. TAK1 of orange-spotted grouper (*Epinephelus coioides)* has also cloned and characterized as a negative regulator in host TLR signaling pathway [16]. In the study of TAK1 of the large yellow croaker (*Larimichthys crocea*), NF-κB luciferase promoter could not be activated by overexpressed TAK1 or TAB1 alone; however, it may be activated by co-expression of the two molecules [17].

Black carp (*Mylopharyngodon piceus*) is an economically important fresh water species, which is one of the "Four Domesticated Fish" in China's freshwater aquaculture. Black carp is subject to a bulk of pathogenic microorganisms in natural and aquacultural conditions such as grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV), however, its innate immune system remains much unknown [18]. In our previous study, bcIKKε, bcTBK1, and bcIRF7 have been cloned and characterized [19–21]. These RLR signaling components of black carp activate interferon production through the mechanisms similar to their mammalian counterparts. At the same time bcTRAF2, bcTRAF3, and bcTRAF6 have been identified. These black carp TRAF members utilize different mechanisms to induce IFN production, which are supposed to be the molecules upstream of TAK1 in host innate immune signaling [22–24]. To illuminate the role of TAK1 in the antiviral innate immune response in black carp, bcTAK1 was cloned and characterized in this paper. In this paper, the data of reporter assay demonstrated that bcTAK1 dramatically improved bcIRF7-mediated IFN inducing ability when co-expressing with bcIRF7 in EPC cells. Furthermore, the data of plaque assay demonstrated clearly that bcTAK1 positively regulates IRF7-mediated antiviral signaling in host innate immune response against both SVCV and GCRV, which elucidates a new mechanism of TAK1 in vertebrates.

2. Materials and methods

2.1. Cells and plasmids

HEK293T, HeLa, *Epithelioma papulosum cyprini* (EPC), *Ctenopharyngodon idella* kidney (CIK), and *Mylopharyngodon piceus* kidney (MPK) cells were kept in the lab [24]. HEK293T and HeLa cells were cultured at 37 °C with 5% CO_2; EPC, CIK, and MPK cells were cultured at 26 °C with 5% CO_2. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfection was done as previously described, calcium phosphate was used for 293T transfection, lipomax (Sudgen, China) was used for EPC transfection (100 ng DNA with 0.3 μl lipomax; efficiency around 80%) and MPK transfection (100 ng DNA with 0.2 μl lipomax; efficiency around 30%) as previously described [25]. pcDNA5/FRT/TO (Invitrogen, USA), pcDNA5/FRT/TO-Flag, pcDNA5/FRT/TO-HA, pRL-TK, Luci-eIFN (for fathead minnow IFN promoter activity analysis), Luci-DrIFNφ1/2/3 (for zebrafish IFNφ1/2/3 promoter activity analysis accordingly), and Luci-bcIFNa (for black carp IFNa promoter activity analysis) were kept in the lab [23]. The recombinant expression vector pcDNA5/FRT/TO-Flag-bcTAK1, pcDNA5/FRT/TO-bcTAK1-Flag, and pcDNA5/FRT/TO-HA-bcTAK1 were constructed by cloning the open reading frame (ORF) of bcTAK1 fused with a Flag or HA tag at its N-terminus/C-terminus into pcDNA5/FRT/TO, respectively.

2.2. Cloning the cDNA of bcTAK1

Degenerate primers (Table 1) were designed to amplify the cDNA of bcTAK1 based on the sequences of TAK1 of grass carp (*C. idella*) (AGI51677.1) and zebrafish (*D. rerio*) (NP_001018586.1). Total RNA was isolated from the spleen of black carp by Trizol (TaKaRa, Japan) and the first-strand cDNA were synthesized by using the Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA). The coding sequence (CDS) was cloned at the first attempt by using the degenerate primers. The amplified fragments were cloned into pMD18-T vector (TaKaRa, Japan) and sequenced by Invitrogen.

Table 1
Primers used in the study.

Primer name	Sequence (5′-3′)	Amplicon length (nt) and primer information
CDS		
bcTAK1-F	ATGTACCCGTTTGAGGAGATAG	1626bp
bcTAK1-R	TCATGATGTGCCCTGTCTC	Partial bcTAK1 CDS cloning
Expression construct		
Flag-bcTAK1-F	ACTGACGGTACCATGTACCCGTTTGAG	
Flag-bcTAK1-R	ACTGACCTCGAGTCATGATGTGCCCTG	FRT-To-Flag-bcTAK1
HA-bcTAK1-F	ACTGACGGTACCATGTACCCGTTTGAG	FRT-To-HA-bcTAK1
HA-bcTAK1-R	ACTGACCTCGAGTCATGATGTGCCCTG	
q-PCR		
bc Q actin-F	TGGGCACCGCTGCTTCCT	
bc Q actin-R	TGTCCGTCAGGCAGCTCAT	*Ex vivo* q-PCR
bc-QTAK1-F1	CTTCGCCAGTTGTCTCGTGT	
bc-QTAK1-R1	GATTGGGTGGTTTGAGGTCC	*Ex vivo* q-PCR

2.3. Virus produce and titration

SVCV (strain: SVCV741) and GCRV (strain: GCRV106) were kept in the lab and propagated in EPC or CIK separately at 26 °C in the presence of 2% fetal bovine serum. EPC or CIK cells were infected with SVCV or GCRV accordingly; the cells and the supernatant media were collected together when the cytopathic effect (CPE) was about 50% and stored at −80 °C. After freezing and thawing for three times, the mixture was used for virus titer mensuration. Virus titers were determined by plaque assay on EPC cells as previously described [24]. Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells and incubated for 2 h at 26 °C. The supernatant was replaced with fresh DMEM containing 2% FBS and 0.75% methylcellulose (Sigma, USA) after incubation. Plaques were counted at day 3 post infection. The virus titer of SVCV was 1×10^7 pfu/ml and titer of GCRV was 1.8×10^7 pfu/ml.

2.4. LPS and poly (I:C) treatment

MPK cells were seeded in 6-well plate (2×10^6 cells/well) 16 h before treatment. Poly (I:C) (Sigma, USA) was used for synthetic dsRNA stimulation, which was heated to 55 °C (in PBS) for 5 min and cooled at room temperature before use. MPK cells were replaced with fresh media containing poly (I:C) at the final concentration of 5 μg/ml, 25 μg/ml, and 50 μg/ml and harvested at different time points (2 h, 8 h, 12 h, 24 h, and 48 h) post treatment. bcTAK1 mRNA level in the MPK cells was determined by quantitative real-time PCR (q-PCR). For LPS (Sigma, USA) treatment, MPK cells in 6-well plate (2×10^6 cells/well) were treated with LPS (1 μg/ml, 10 μg/ml, and 50 μg/ml) separately and harvested at different time points (2 h, 8 h, 12 h, 24 h, and 48 h) post stimulation as above.

2.5. Quantitative real-time PCR

The relative bcTAK1 mRNA level in the MPK cells was determined by quantitative real-time PCR. The primers for bcTAK1 and β-actin (as

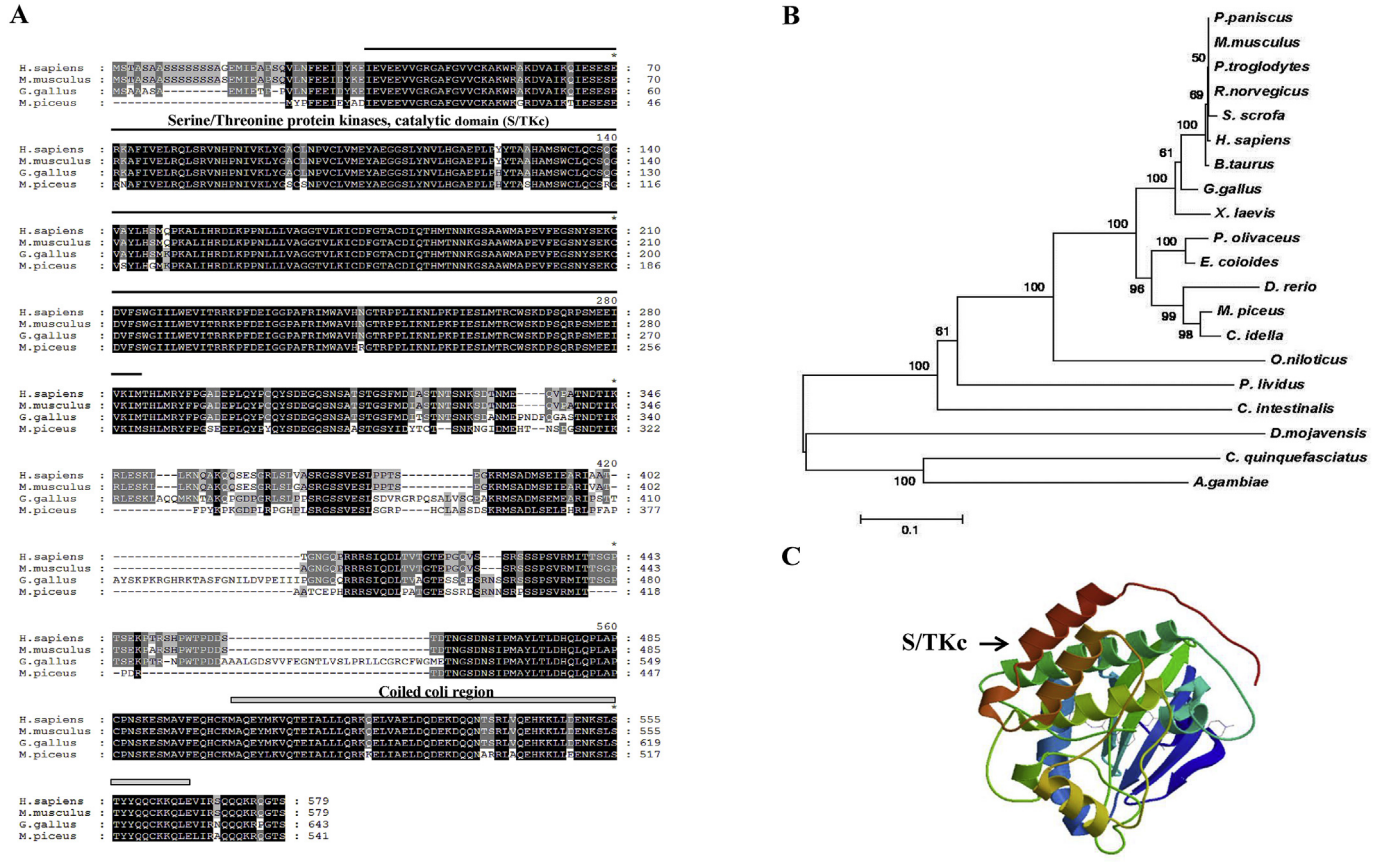

Fig. 1. Evolution of vertebrate TAK1.(A). Comparisons of bcTAK1 with other vertebrate TAK1 protein sequences by using MEGA 6.0 program and GeneDoc program, which including: *H. sapiens* (AAV38460.1), *M. musculus* (NP_033342.1), *G. gallus* (XP_419832.3), and *M. piceus*. The protein domains were predicted by CDS (Conserved Domain Search) of NCBI (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi), and Simple Modular Architecture Research Tool (SMART) (http://smart.emble-heidelberg.de). (B). By using MEGA 5.0 program, maximum likelihood phylogenetic tree was generated from vertebrate TAK1 of different species which include (GenBank accession number): *E. coioiaes* (AGQ48129.1), *P. olivaceus* (AJE25832.1), *D. rerio* (NP_001018586.1), *C. idella* (AGI51677.1), *M. musculus* (NP_033342.1), *B. taurus* (NP_001075064.1), *H. sapiens* (AAV38460.1), *X. laevis* (NP_001084359.1), *C. quinquefasciatus* (XP_001848119.1), *S. scrofa* (APX52954.1), *P. lividus* (ABF82443.1), *R. norvegicus* (NP_001101390.2), and *C. intestinalis* (NP_001071829.1). The bar stands for scale length and the numbers on different nodes stand for bootstrap value. (C). The predicted protein structure of bcTAK1 (by SWISS-MODEL; https://www.swissmodel.expasy.org/).

internal control) were listed in Table 1. The q-PCR program was: 1 cycle of 50 °C/2min, 1 cycle of 95 °C/10min, 40 cycles of 95 °C/15s, 60 °C/1min, followed by dissociation curve analysis (60 °C-95 °C) to verify the amplification of a single product. The threshold cycle (CT) value was determined by using the manual setting on the Applied Biosystems Fast 7500 Real-Time PCR System (ABI, USA) and exported into a Microsoft Excel spreadsheet for subsequent data analysis where the relative expression ratios of target gene in treated groups versus those in control group were calculated by $2^{-\triangle\triangle CT}$ method [26]. The data were analyzed by two-tailed Student's t-test with the GraphPad Prism 4.0 software (GraphPad Prism, USA).

2.6. Luciferase reporter assay

EPC cells in 24-well plate were co-transfected with pRL-TK (25 ng), Luci-eIFN (Luci-bcIFNa or Luci-DrIFNφ1/2/3) (250 ng), pcDNA5/FRT/TO-Flag-bcTAK1, and/or pcDNA5/FRT/TO-HA-bcIRF7. For each transfection, the total amount of plasmid DNA (425 ng) was balanced with the empty vector. The cells were harvested and lysed by renilla luciferase lysis buffer (Promega, USA) on ice at 24 h post transfection. The centrifuged supernatant was used to measure firefly luciferase and renilla luciferase activities according to the instruction of the manufacturer (Promega, USA) as described previously [25].

2.7. Immunoblotting

HEK293T cells in 6-well plate (2×10^6) were transfected with plasmid expressing bcTAK1 (pcDNA5/FRT/TO-Flag-bcTAK1, pcDNA5/FRT/TO-bcTAK1-Flag, pcDNA5/FRT/TO-HA-bcTAK1) or the empty vector separately. EPC cells in 6-well plate (2×10^6) were transfected with pcDNA5/FRT/TO-Flag-bcTAK1, pcDNA5/FRT/TO-bcTAK1-Flag or the empty vector separately. Transfected cells were harvested at 48 h post-transfection and lysed for immunoblot (IB) assay as previously described [20]. Briefly, the whole cell lysates were isolated by 10% SDS-PAGE and transferred to PVDF membrane. The transferred membranes were probed with mouse monoclonal *anti*-Flag antibody (1:3000; Sigma, USA) or mouse monoclonal *anti*-HA antibody (1:4000; Sigma, USA), which were followed by the incubation with goat-*anti*-mouse IgG (1:30000; Sigma, USA). The target proteins were visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma, USA).

2.8. Immunofluorescence microscopy

HeLa cells, EPC cells, and MPK cells in 24-well plate were transfected with plasmid expressing bcTAK1 or the empty vector separately. The transfected cells were fixed with 4% (v/v) paraformaldehyde at 24 h post-transfection. The fixed cells were permeabilized with Triton X-100 (0.2% in PBS) and used for immune-fluorescent staining as

Table 2
Comparison of bcTAK1 with other vertebrate TAK1 (%).

Species	Full-length sequence	
	Similarity	Identity
Mylopharyngodon. piceus	100	100
Mus. musculus	74.7	80.8
Homo. sapiens	59.6	64.4
Rattus. norvegicus	71.7	77.4
Pan. paniscus	71.6	77.4
Pan. troglodytes	71.6	77.4
Sus. scrofa	73.9	80.0
Bos. taurus	74.3	80.5
Gallus. gallus	70.7	76.1
Xenopus. laevis	73.2	78.6
Epinephelus. coioides	84.4	87.9
Paralichthys. olivaceus	83.7	87.9
Danio. rerio	82.0	85.2
Ctenopharyngodon. idella	97.2	98.5
Oreochromis. niloticus	61.7	70.8
Ciona. intestinalis	47.4	58.6
Paracentrotus. lividus	42.3	53.4
Culex. quinquefasciatus	36.4	46.4
Anopheles. gambiae	38.7	51.1
Drosophila. melanogaster	30.4	43.9

previously described [24]. Mouse monoclonal *anti*-Flag antibody (Sigma, USA) was probed at the ratio of 1:300; Alexa 594-conjugated secondary antibody (Invitrogen, USA) was probed at the ratio of 1:200 and Alexa 488-conjugated secondary antibody (Invitrogen, USA) was probed at the ratio of 1:800; DAPI was used for nucleus staining.

2.9. Statistics analysis

For the statistics analysis of the data of q-PCR, luciferase reporter assay and viral titer measurement, all data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (+SEM) of three independent experiments. Asterisk (*) stands for $p < 0.05$. Two-tailed Student's *t*-test was used for all statistical analyses with the GraphPad Prism 4.0 software (GraphPad Prism, USA).

3. Results

3.1. Molecular cloning and sequence analysis of bcTAK1

To learn the role of bcTAK1 in black carp, the cDNA of TAK1 was cloned from the liver of black carp and the coding sequence of bcTAK1 consists of 1626 nucleotides (NCBI accession number: MH114078). Initial sequence analysis of bcTAK1 cDNA (online tools of ExPAsy) predicts that bcTAK1 contains 541 amino acid residues, including an S/TKc domain (12–260) and a coiled coil domain (465–528). bcTAK1 has a calculated molecular weight of 75 kDa and an isoelectric point of 7.55. To gain insight into TAK1 evolution, amino acid sequence of bcTAK1 has been subjected to multiple alignments with those of TAK1 from human (*H. sapiens*), mouse (*M. musculus*), and chicken (*G. gallus*). The data demonstrates that TAK1 is a conserved protein in vertebrates, especially its N-terminal S/TKc domain and C-terminal coiled coil motif (Fig. 1A and C). Phylogenetic analysis has been applied to bcTAK1 and TAK1 proteins of other known species (Fig. 1B). bcTAK1 shares high amino acid sequence similarity with grass carp (*C. idella*) TAK1 (98.5%) and is clustered tightly with grass carp TAK1, which correlates with the closest genetic relationship of these two cyprinid fishes (Fig. 1B and

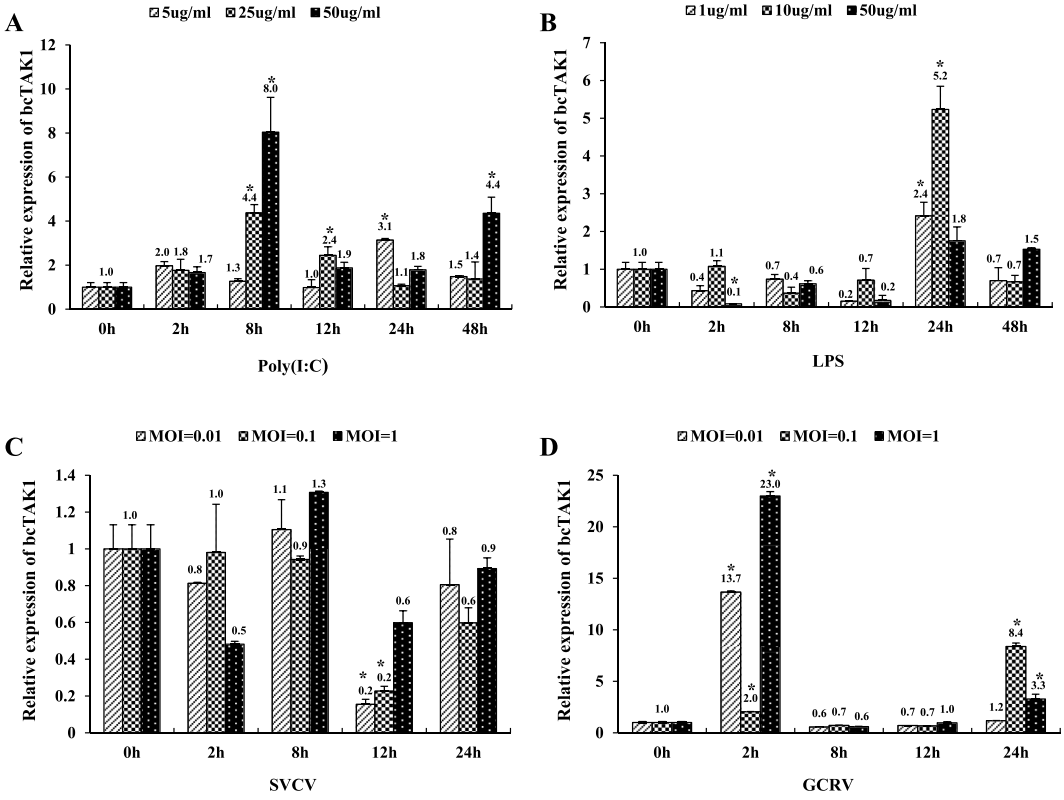

Fig. 2. Expression of bcTAK1 in response to different stimuli. MPK cells in 6-well plate (2×10^6 cells/well) were treated with poly (I:C) (A) or LPS (B) at indicated concentration separately; or infected with SVCV (C) or GCRV (D) at indicated MOI separately. The cells were harvested at indicated time points post stimulation separately and used for RNA isolation. The relative bcTAK1 mRNA level was examined by q-PCR. The numbers above the error bars stand for average bcTAK1 mRNA level, error bars denote standard deviation and asterisk (*) stands for $p < 0.05$.

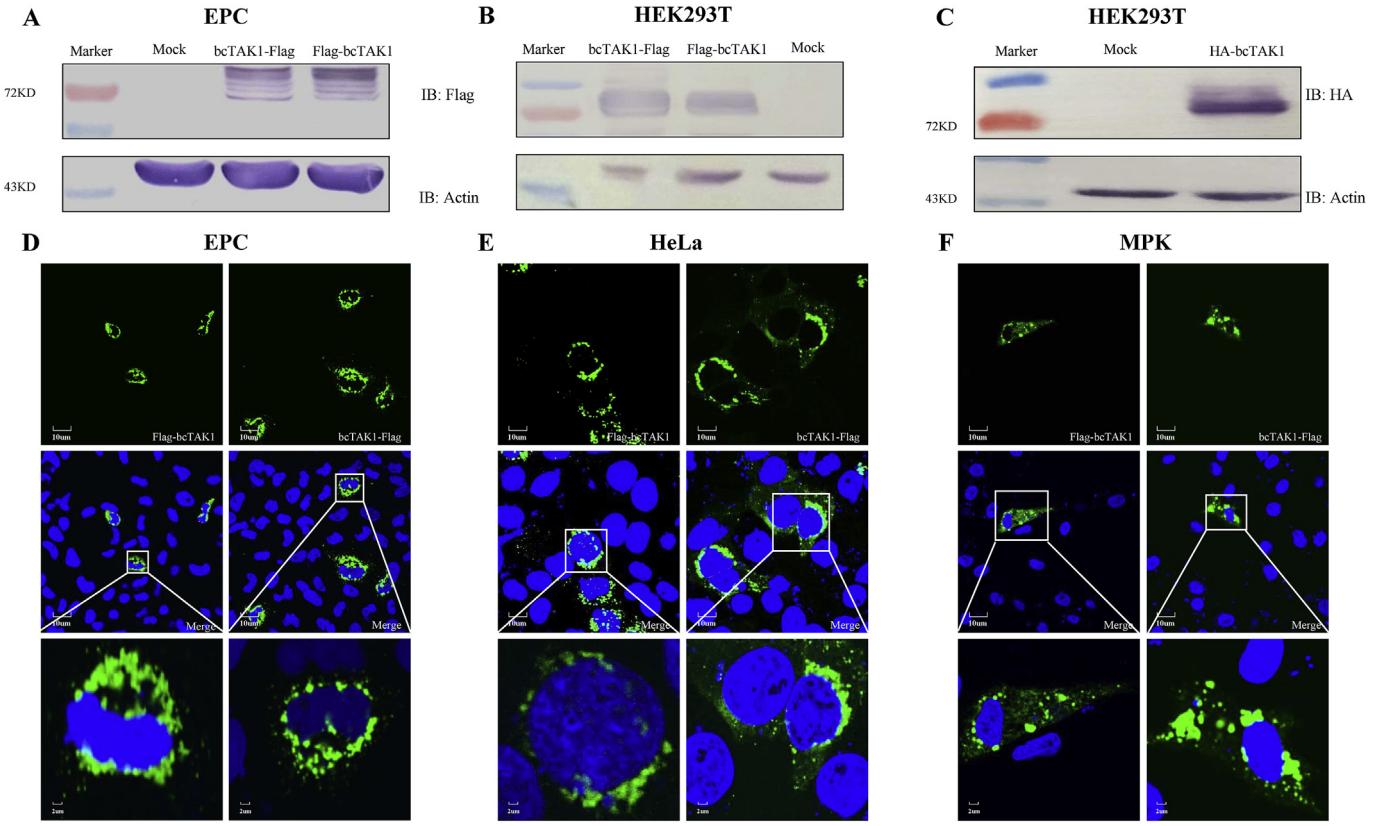

Fig. 3. Protein expression and subcellular distribution of bcTAK1. EPC (A) or 293T (B and C) cells were transfected with pcDNA5/FRT/TO-Flag-bcTAK1, pcDNA5/FRT/TO-bcTAK1-Flag, pcDNA5/FRT/TO-HA-bcTAK1, or the empty vector separately. The transfected cells were harvested and lysed at 48 h post transfection. The whole cell lysates were used for immunoblot (IB) assay in which bcTAK1 were detected by *anti*-Flag antibody or *anti*-HA antibody. Mock: 293T or EPC cells transfected with empty vector, Flag-bcTAK1: pcDNA5/FRT/TO-Flag-bcTAK1, bcTAK1-Flag: pcDNA5/FRT/TO-bcTAK1-Flag, HA-bcTAK1: pcDNA5/FRT/TO-HA-bcTAK1. EPC cells (D), HeLa cells (E), or MPK cells (F) were transfected with pcDNA5/FRT/TO-Flag-bcTAK1, pcDNA5/FRT/TO-bcTAK1-Flag, or the empty vector separately. The transfected cells were fixed at 36 h post transfection and used for immunofluorescence staining according to the methods. TAK1 (green) indicates intracellular expression of bcTAK1, DAPI (blue) indicates nucleus; the bars stand for the scale of 2 μm or 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2).

3.2. bcTAK1 expression ex vivo in response to different stimulations

To learn bcTAK1 mRNA profile during host innate immune response, MPK cells were subject to different stimuli and bcTAK1 transcription was examined by qPCR. The data showed that there was a significant change in the mRNA level of bcTAK1 before and after stimulation. The mRNA level of bcTAK1 in MPK cells increased significantly right after poly (I:C) treatment and decreased, then increased again within the first 48 h post poly (I:C) treatment. The maximum relative mRNA level of bcTAK1 in the stimulated MPK cells (50 μg/ml dosage, 8 h point) was up to 8-fold of that of control cells. In addition, the mRNA level of bcTAK1 was back up to 4.4 (50 μg/ml dosage, 48 h point) after a period of decline (Fig. 2A). However the mRNA level of bcTAK1 was decreased right after LPS treatment (2 h) and slightly increased at 24 h point, and dropped down again at 48 h point. The maximum relative mRNA level of bcTAK1 in the stimulated MPK cells (10 μg/ml dosage, 24 h point) was up to 5.2-fold of that of control cells and the minimum relative mRNA level of bcTAK1 in the stimulated MPK cells was only 10% of that of control (50 μg/ml dose, 2 h point) (Fig. 2B).

In SVCV infected MPK cells, mRNA level of bcTAK1 was decreased during the first 24 h post infection, in which the minimum relative mRNA level of bcTAK1 MPK cells was only 20% of that of control (0.01 MOI, 12 h point) (Fig. 2C). However, bcTAK1 transcription in MPK cells was obviously enhanced by GCRV right after infection (2 h) and the maximum relative mRNA level of bcTAK1 (1 MOI, 2 h) was up to 23-fold of that of control cells. bcTAK1 expression decreased from 8 h post infection (hpi) and increased again at 24 hpi (Fig. 2D). The date showed that GCRV and SVCV induce different bcTAK1 transcription in MPK cell.

3.3. Protein expression and subcellular distribution of bcTAK1

EPC cells or HEK293T cells were transfected with plasmids expressing bcTAK1 and used for immunoblotting (IB) assay to investigate the protein expression of bcTAK1, in which mouse *anti*-Flag antibody or *anti*-HA antibody were used to detect the exogenous bcTAK1. The specific bands of ~75 KDa were detected in the whole cell lysate of both EPC cells and HEK293T cells transfected with Flag-bcTAK1, bcTAK1-Flag or HA-bcTAK1 but not in the empty vector-transfected cells, which matched the predicted molecular weight of this fish protein (Fig. 3A, B and C). It is interesting that several bands representing bcTAK1 were detected in the IB assay. It was speculated that bcTAK1 proteins were modified post translation, possibly modified with glycosylation.

To determine the subcellular location of bcTAK1, EPC cells, HeLa cells, or MPK cells were transfected with plasmids expressing Flag-bcTAK1 or bcTAK1-Flag separately and used for immunofluorescence staining (IF). The IF data in both EPC cells, HeLa cells, and MPK cells showed clearly that bcTAK1 expression region (green) surrounded tightly the nucleus (blue), which demonstrated that bcTAK1 was mainly distributed in cytoplasm. Especially, brilliant green dots representing

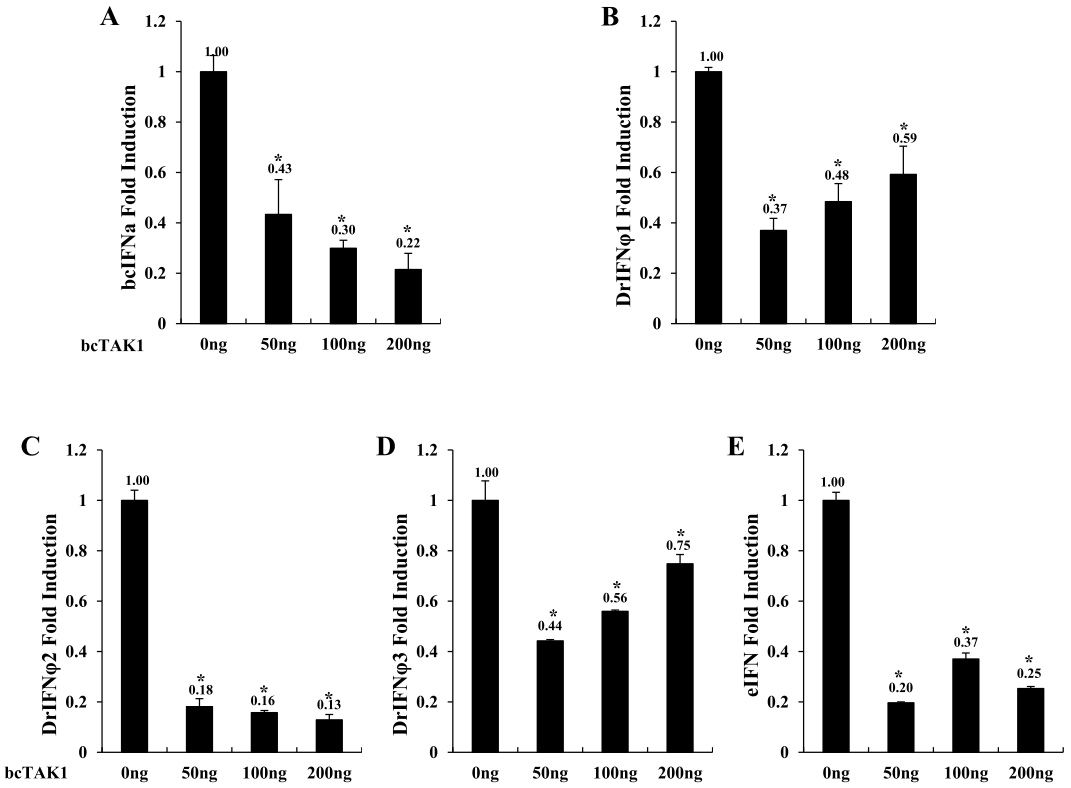

Fig. 4. IFN Signaling induced by bcTAK1.EPC cells in 24-well plate were co-transfected with pRL-TK, Luci-bcIFNa (Luci-eIFN, Luci-DrIFNφ1, Luci-DrIFNφ2, and Luci-DrIFNφ3), bcTAK1, or the empty vector separately and applied to luciferase reporter assay according to methods. A. Black carp IFNa promoter induction by bcTAK1. B. Zebrafish IFNφ1 promoter induction by bcTAK1. C. Zebrafish IFNφ2 promoter induction by bcTAK1. D. Zebrafish IFNφ3 promoter induction by bcTAK1. E. Fathead minnow IFN promoter induction induced by bcTAK1. The error bars represent the standard deviation and data represent three independent experiments. Asterisk (*) stands for $p < 0.05$. bcTAK1: pcDNA5/FRT/TO-Flag-bcTAK1.

bcTAK1 were widely scattered in the cytoplasmic region, which implied that the aggregation of bcTAK1 molecules or the interaction of bcTAK1 with other molecules (Fig. 3D, E and F).

3.4. IFN signaling regulated by bcTAK1

To investigate the effect of bcTAK1 on IFN signaling, EPC cells were transfected with plasmid expressing bcTAK1 and use for dual luciferase reporter assay. In general, overexpression of bcTAK1 in EPC cells suppressed induced transcription of bcIFNa, DrIFNφ1, DrIFNφ2, DrIFNφ3, and eIFN (Fig. 4). In the reporter assay, bcTAK1 down-regulated bcIFNa transcription in a dose dependent manner and the biggest reduction of bcIFNa transcription was up to 78%. Similar down-regulation trend was seen in the data of DrIFNφ2 group, in which the biggest reduction of DrIFNφ2 transcription was up to 87% (Fig. 4A and C). However, induced DrIFNφ1 transcription was 63% of that of control when the cells were transfected with 50 ng bcTAK1 and the reduction of induced DrIFNφ1 transcription decreased when bcTAK1 input increased, and similar phenomenon was seen in DrIFNφ3 data (Fig. 4B and D). It was interesting that the reduction of induced eIFN transcription varied as bcTAK1 input increased: 80% for 50 ng input, 63% for 100 ng, and 75% for 200 ng (Fig. 4E).

3.5. bcTAK1 up-regulated bcIRF7-mediated antiviral signaling

Previous study demonstrates that bcIRF7 plays an important role in host antiviral innate immune response against both GCRV and SVCV [20]. In mammal, IRF7 needs to be phosphorylated before it is activated. To explore whether TAK1 can activate IRF7 as a kinase or not, bcTAK1 and/or bcIRF7 were expressed in EPC cells, which were used for reporter assay. It was really interesting that bcIRF7-mediated induction of both DrIFNφ1, DrIFNφ3, and bcIFNa were obviously enhanced by bcTAK1, especially the fold induction of DrIFNφ1 (bcIRF7-mediated induction of DrIFNφ1 was 16.23-fold of control, however, it increased to 227.21-fold when co-expressed with bcTAK1) and bcIFNa (bcIRF7-mediated induction of bcIFNa was 6.30-fold of control, however, it increased to 230.43-fold when co-expressed with bcTAK1), which suggested that bcTAK1 positively regulated bcIRF7-mediated IFN signaling (Fig. 5A, B and C).

To further explore whether bcTAK1 regulates the antiviral activity of bcIRF7, EPC cell were transfected with plasmid expressing bcTAK1 and/or plasmid expressing bcIRF7, then subject to GCRV (MOI = 1, MOI = 0.1, MOI = 0.01) and SVCV (MOI = 1, MOI = 0.1, MOI = 0.01) infection. In GCRV infected group, bcTAK1 showed little antiviral ability in contrast to the control group, while overexpressed bcIRF7 enhanced the antiviral activity of EPC cells against GCRV. It was interesting that the viral titer of EPC cells expressing bcIRF7 alone was higher than the cells expressing both bcTAK1 and bcIRF7, which demonstrated that bcIRF7-mediated antiviral activity in EPC cells against GCRV was obviously enhanced when co-expressed with bcTAK1 (Fig. 5D). Similar to the data of GCRV group, SVCV titer of EPC cells expressing bcIRF7 alone was higher than the cells expressing both bcTAK1 and bcIRF7, which demonstrated that bcIRF7-mediated antiviral activity in EPC cells against SVCV was obviously enhanced when co-expressed with bcTAK1 (Fig. 5E). Thus, our data demonstrated clearly that bcTAK1 positively regulated bcIRF7-mediated antiviral activity, which correlated with the luciferase reporter assay results.

4. Discussion

Most studies of TAK1 focus on its function in the human cancer and most part of these researches are conducted by mutating or silencing

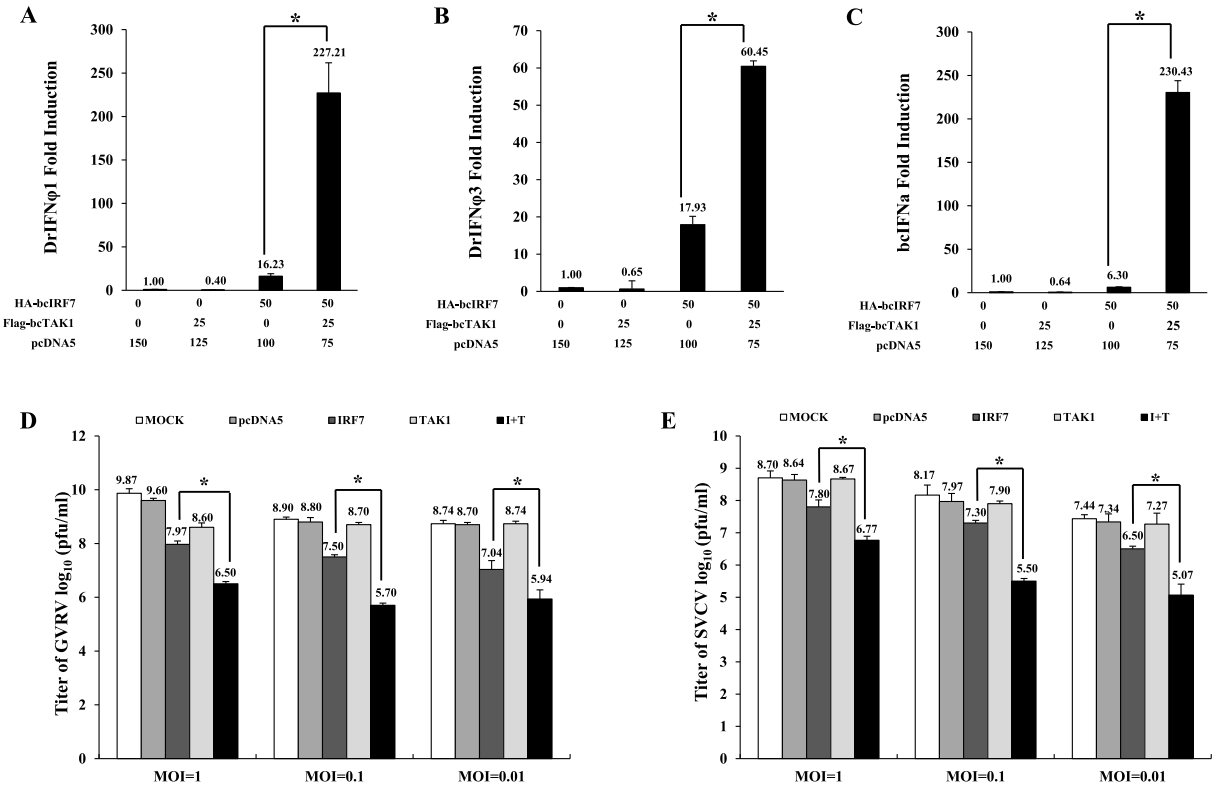

Fig. 5. bcTAK1 up-regulated bcIRF7-mediated antiviral signaling.A, B, and C. EPC cells in 24-well plate (2×10^5 cells/well) were co-transfected with pRL-TK, Luci-DrIFNφ1/Luci-DrIFNφ3/Luci-bcIFNa, bcTAK1, and/or bcIRF7, and applied to reporter assay. HA-bcIRF7: pcDNA5/FRT/TO-HA-bcIRF7; Flag-TAK1: pcDNA5/FRT/TO-Flag-bcTAK1; pcDNA5: pcDNA5/FRT/TO. D and E. EPC cells in 24-well plate (2×10^5 cells/well) were co-transfected with bcTAK1 and/or bcIRF7. The transfected cells were infected with GCRV or SVCV at 24 h post-transfection and the virus titers in the supernatant media were determined by plaque assay at 48 h post-infection. The numbers above the error bars stand for average virus titer and error bars represent the standard deviation. bcTAK1: pcDNA5/FRT/TO-Flag-bcTAK1; bcIRF7:pcDNA5/FRT/TO-HA-bcIRF7; Mock: cells without transfection; pcDNA5: cells transfected with the empty vector; IRF7: pcDNA5/FRT/TO-HA-bcIRF7; TAK1:pcDNA5/FRT/TO-Flag-bcTAK1; I + T: pcDNA5/FRT/TO-HA-bcIRF7 and pcDNA5/FRT/TO-Flag-bcTAK1; Numbers stand for the average virus titer (Log_{10} pfu/ml).

TAK1 gene to explore its role in the proliferation of tumor cells. Embryonic lethality with defects in vascularization, angiogenesis and smooth muscle formation was found in mouse with mutated TAK1 [27,28]. In the studies of parasite (*Caenorhabditis elegans*), clawed frog (*Xenopus*), and zebrafish (*Danio rerio*), TAK1 mutation or knock-down impacted endoderm specification, axis formation, and vasculature development [11]. Previous studies have shown that TAK1 is involved in NF-κB activation in TLR family signaling pathways, in which MyD88 serves as an adaptor for IL-1 receptor-associated kinase (IRAK) 1 and 4 [29–31]. The interaction between hyperphosphorylated IRAK1 and TRAF6 results in the oligomerization of TRAF6, which leads to IRAK1/TRAF6 complex to dissociate from the receptor and associate with TAK1 [31,32]. Ubc13/Uev1A-mediated polyubiquitination of TRAF6 is considered to be crucial for the association with TAB2 (or TAB3), which links TAK1 activation [30,33]. TAK1/TAB complex triggers the phosphorylation and degradation of IκB, which finally results in the activation of NF-κB and pro-inflammatory cytokines production [34,35]. Additionally, it has been reported that activated IRAK1/TRAF6 complex by TLR7/9 stimulates IKKα phosphorylation and activation of IRF7, allowing the production of type I IFNs [36–38].

In this study, TAK1 homologue has been characterized from black carp and bcTAK1 possesses two conserved functional domains like its mammalian counterpart, including an N-terminal S/TKc domain and a C-terminal coiled coli region. In mammals, S/TKc domain is necessary for TAK1 to activate IKKs and MAPKs and autophosphorylation of two threonine residues in the activation loop of TAK1 was necessary for TAK1 activation [39,40]. Coiled coli region is the binding region of TAB2, which interacts with TAK1 and, thereby, mediates its association with TRAF6 [30,34]. Phylogenetic tree analysis in this paper further indicates that bcTAK1 is well clustered with fish TAK1 and closely related to that of grass carp and zebrafish. bcTAK1 migrated around 75 KDa in the immunoblot assay, in which several specific bands representing bcTAK1 were detected (Fig. 3). It is interesting that the asparagine residues of 181, 318, 406, 425, and 513 sites in bcTAK1 are located in the conserved N-linked glycosylation motif (N-X-S/T), which suggests that bcTAK1 is modified with N-linked glycosylation. However, whether this protein possesses N-linked glycosylation needs further exploration.

In RLR pathway, RIG-I/MDA5 recognize viral dsRNA in the cytosol and signal through the adaptor protein MAVS, which localizes mainly on the outer mitochondrial membrane [41]. Triggered by RIG-I/MDA5, MAVS recruits TBK1 and IKKε to phosphorylate and activate IRF3 and IRF7, which translocate into the nucleus to regulate the transcription of type I IFNs and IFN-stimulated genes (ISGs) [42,43]. TRAF members such as TRAF2 and TRAF6 are considered to be involved into this process with different mechanisms [44]. In our previous study, both bcTRAF2 and bcTRAF6 showed little activity of either zebrafish IFN promoter or fathead minnow IFN promoter induction in reporter assay; however, both bcTRAF2 and bcTRAF6 obviously improved bcMAVS-mediated IFN induction [21,23]. In this study, bcTAK1 presented little activity of IFN promoter induction in reporter assay; however, bcIRF7-mediated IFN signaling and antiviral activity was much improved when the cells co-expressed bcIRF7 and bcTAK1. It is speculated that bcTAK1 has been recruited into host MAVS/TRAF6/IRF7 signaling initiated by RNA virus infection such as GCRV (double stranded RNA virus) and SVCV (single stranded RNA virus) and the mechanisms by which they

infect black carp may be different, which lead to the different antiviral innate immune mechanism by the host cells. IRF7 has been considered as the master regulator of type-I IFN-dependent immune responses in human and mammals; however, no reporter before showed that TAK1 positively regulated IRF7-mediated antiviral IFN signaling. Although our data demonstrates the synergistic relationship between bcTAK1 and bcIRF7 for the first time in vertebrates, the mechanism behind these two molecules during host innate immune response initiated by GCRV or SVCV remains unknown. Additionally, whether and how bcTRAF6 is involved into the coordination between bcTAK1 and bcIRF7 needs to be further explored.

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