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Short communication

Molecular identification and expression analysis of TAB1 from orange-spotted grouper (*Epinephelus coioides*)

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ABSTRACT

Transforming growth factor-β activated kinase 1 (TAK1) is a crucial signal transducer in multiple signaling pathways. TAK1 binds TAB1, TAB2, and TAB3, which act as activators and adaptors that specifically regulate the activation of TAK1. To date, the role of TABs is largely unknown in fish. In the present study, a TAB1 cDNA sequence was identified in grouper (*Epinephelus coioides*), and designated EcTAB1. The full-length open reading frame of EcTAB1 is 1, 521 bp; it encodes 506 amino acids that contains an N-terminal PP2C domain. Many important functional sites in mammalian TAB1 were conserved in TAB1 from grouper and from other fish. Multiple sequence alignment showed that EcTAB1 protein shared high sequence identity with TAB1 of other fish, especially with *Stegastes partitus* (95% identity). TAB1 was clustered into the same subgroup with other fish TAB1 in the phylogenetic tree. Tissue expression analysis indicated that TAB1 was widely distributed in different tissues. After infection with *Cryptocaryon irritans*, EcTAB1 expression was up-regulated in the infection site (gills). Besides, EcTAB1 was expressed throughout the grouper spleen (GS) cells and significantly enhanced the activation of NF-κB.

1. Introduction

Transforming growth factor-β-activated kinase 1 (TAK1), also called MAP3K7, can be activated by a variety of stimuli including cytokines (e.g. TGF-β, IL-1β and TNF-α), bone morphological protein, Toll-like receptor (TLR) ligands, and adaptive immunity-related B and T cell receptor ligands (Ajibade et al., 2013; Dai et al., 2012). The function of TAK1 depends on the formation of a TAK1 complex, which is mediated by association with its partners TAK1-binding proteins 1 (TAB1), TAB2, or TAB3 (Cheung et al., 2004; Shibuya et al., 1996; Takaesu et al., 2000).

TAB1 was initially identified as a partner protein of TAK1 using the yeast two-hybrid system. Overexpression of TAB1 increased the kinase activity of TAK1 in response to TGF-β stimuli (Shibuya et al., 1996). Later studies have shown that TAB1 is a pseudo-substrate, which contains a conserved protein phosphatase 2Cα (PP2C)-like domain in the N-terminal region, but lacks phosphatase activity (Conner et al., 2006). TAB1 constitutively interacts with the N-terminal catalytic domain of TAK1, and directly induces TAK1 kinase activity in resting cells. TAB2 and TAB3 bind TAK1 only after stimulation (Ishitani et al., 2003; Sakurai et al., 2002). Unlike the TAK1 activator TAB1, TAB2 does not directly activate TAK1; it acts as a TAK1 adaptor by linking the C-terminal of TAK1 to the upstream elements of the signaling pathway (Takaesu et al., 2000). TAB3 compensates for the loss of TAB2 and functions as a mediator of TAK1 kinase activity (Cheung et al., 2004). TAB1 directly binds p38α, in addition to TAK1, and mediates p38α auto-phosphorylation and activation, which is considered as an alternative p38α activation mechanism (Ge et al., 2002). However, Cheung et al. (2003) have indicated that the interaction of TAB1 with p38α directly phosphorylated it at Ser423 and Thr431 by p38α, which then blocked the activation of TAK1.

Previous studies have indicated that K63-linked poly-ubiquitination at the residue K158 of TAK1 is required for its activation following stimulation (Ajibade et al., 2013). In NF-κB and MAPK signaling pathways, triggered by IL-1β, TAK1 activation requires ubiquitination by TRAF6 through two intermediary factors, TRAF6-regulated IKK activator 1 (TRIKA1) and TRIKA2 (Deng et al., 2000; Wang et al., 2001). TRIKA1 consists of two ubiquitin-conjugating enzymes Ubc13 and the

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Ubc-like protein Uev1A. TRIKA2 is a ternary complex composed of TAK1, TAB1, and TAB2. When activated by the upstream signal molecules interleukin-1 receptor-associated kinases (IRAKs), TRAF6 catalyzes self-ubiquitination through Ubc13 and Uev1A (Qian et al., 2001). TAB2 bridges activated TRAF6 to TAK1 by binding to its K63-linked polyubiquitin, and leads to the K63-linked polyubiquitination of TAK1 and its activation. The activated TRAF6-TAB2-TAK1-TAB1 complex interacts with downstream signal molecules such as IKKs and MAPKs, and activates them, which then results in nuclear translocation of NF-κB, p38, and JNK (Matsuzawa et al., 2005). In TNF signaling pathway, TRAF2 is phosphorylated after TNF stimulation, and promotes K63-linked ubiquitination, which facilitates the formation of the TRAF2-TAB2-TAK1-TAB1 complex, and the recruitment of IKKs to the TNF receptor (Hong et al., 2007).

Although TABs play crucial roles in mammals, the function of these molecules is largely unknown in aquatic animals. Zhao et al. (2014) reported that *Ichthyophthirius multifiliis* infection could significantly increase the expression of *Ctenopharyngodon idella* TAB1 and TAB2. Yin et al. (2016) recently identified TAB1 from *Branchiostoma belcheri*, and detected its expression after LPS stimulation. To the best of our knowledge, no other study has reported the function of piscine TABs. In the present study, we identified grouper TAB1 cDNA sequence (EcTAB1), analyzed the structure conservation of this molecule, detected the expression pattern in healthy fish and in fish infected with *Cryptocaryon irritans*. Finally, the signal transduction function of EcTAB1 was studied in grouper spleen (GS) cells.

2. Materials and methods

2.1. Identification of TAB1 sequences

2.1.1. PCR amplification

The degenerate primers TAB1F1 and TAB1R5 (Supplementary Table S1) were designed based on the conserved regions of TAB1 from *Danio rerio* (XM_002662240), *Oreochromis niloticus* (XM_005464854), *C. idella* (KJ184547), *Takifugu rubripes* (XM_003978664) and *Oryzias latipes* (XM_004066116). The primers TAB1F1 and TAB1R5 were used to amplify a partial sequence of grouper TAB1. Gene-specific primers RTAB1R1/RTAB1R2 and RTAB1F1/RTAB1F2 (Supplementary Table S1) were used to obtain the 5′ and 3′-terminal end sequence with a SMARTer RACE cDNA Amplification Kit (Clontech, USA), following the manufacturer's protocols. Subsequently, the primers TAB1FLF/R (Supplementary Table S1) were used to clone the complete open reading frame (ORF) of EcTAB1, which was used as template to construct the expression vectors detailed in section 2.3.1. All the PCRs were performed using PrimeSTAR® HS DNA polymerase (Takara, Japan). Target PCR products were purified using an E.Z.N.A.® Gel Extraction Kit (Omega, USA). Subsequently, purified PCR products were cloned into the pEASY®-Blunt Simple Cloning vector (TransGen Biotech, China), and sequenced by Life Technologies Corporation Ltd (USA).

2.1.2. Bioinformatics analysis

Multiple sequence alignments were performed using Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). The theoretical isoelectric point and the molecular weight were calculated using the Compute pI/MW Tool (http://www.expasy.org/tools/pi_Tool. html). Protein structure analysis was performed using the SMART program (http://smart.emblheidelberg.de/). Phylogenetic tree analysis was performed using the MEGA 5.04 program.

2.2. Gene expression analysis

2.2.1. Experimental animals and tissue sampling

Healthy orange-spotted grouper (95–110 g) were obtained from a governmental hatchery (Marine Fisheries Development Center of Guangdong Province, China). The gills and mucus from 10 fish were analyzed using a light microscope to ensure that there was no parasite infection. The serum of these fish was tested for no immobilization of *C. irritans*. Fish were then acclimatized at 27 °C with re-circulating seawater for 2 weeks, and fed twice a day with a commercial grouper diet.

C. irritans was originally isolated from infected pompano *Trachinotus ovatus*, and then identified according to morphological features (Dan et al., 2006). They were propagated in our laboratory using *T. ovatus* as host, following the method described by Dan et al. (2006).

For experimental infections, 80 groupers were divided randomly into *C. irritans*-infected group and uninfected control group. Forty fish in the infection group were exposed to *C. irritans* (30,000 theronts per fish) for 2 h; after this time the seawater was continuously circulated. The uninfected controls were treated the same way as the infection group, with the exception of parasite exposure. Two days later, the gills and mucus from five infected grouper were sampled to ensure that fish were infected successfully. At 6 h, 12 h, 1 d, 2 d, and 3 d post infection, gills (local infection site) and spleen (systemic immune organ) were collected from both infected group and uninfected group; five fish were sampled at each time point. The thymus, gill, head kidney, skin, muscle, liver, spleen, trunk kidney, and brain were sampled from three healthy groupers, and used to analyze the tissue expression patterns of target genes. All the protocols about the care of fish were approved by the Institutional Animal Care and Use Committees at Sun Yat-sen University. All samples were snap-frozen in liquid nitrogen, and then stored at −80 °C until RNA isolation.

2.2.2. RNA extraction and cDNA synthesis

Total RNA was isolated using Trizol™ Reagent (Invitrogen, USA), following the manufacturer's instructions. The integrity of the extracted RNA was determined by 1% agarose gel electrophoresis; RNA purity was analyzed using OD260/280 measurements. One μg of total RNA was incubated with RNase-free DNase I (Fermentas, Canada) at 37 °C for 30 min, and then used to synthesize the first strand cDNA using ReverTra Ace-α reverse transcriptase (Toyobo, Japan).

2.2.3. Gene expression profiling

Real-time PCR was used to detect the expression of EcTAB1. The primers QTAB1F1/R1 and β-actinF1/R1 (Supplementary Table S1) were designed using Beacon Designer 7.80. Real-time PCR was performed using a Roche LightCycler® 480 Real-time PCR Detection System (Roche, Switzerland) with SYBR Green Real-time PCR Master Mix (Toyobo). The PCR cycles were as follows: 94 °C for 2 min, 40 cycles at 94 °C for 15 s, 57 °C for 15 s, and 72 °C for 20 s. The specificity of the PCR products was assessed by melting curve analysis and sequencing. Each sample was tested in triplicate. The mRNA expression level of the target gene was calculated relative to the reference gene, β-actin, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.3. Functional analysis

2.3.1. Plasmids construction

The ORF of EcTAB1 was amplified using the primers with (RPTAB1F1/R1) or without (RPTAB1F1/R2) stop codon, which contain restriction enzyme sites *Eco*RI and *Xho*I at the 5′ terminal, respectively (Supplementary Table S1). After double enzyme digesting, target gene sequences were subcloned into pcDNA3.1-GFP (Li et al., 2016) using T4 DNA ligase (Takara). All the plasmids were then transformed into *Escherichia coli* TG1 and sequenced. Subsequently, plasmids were extracted from TG1 using an E.Z.N.A.® Endo-free Plasmid Mini Kit (Omega) according to the manufacturer's instructions.

2.3.2. Cells and transfection

GS cells, kindly provided by Pro. Qiwei Qin (College of Marine Sciences, South China Agricultural University), were cultured in L-15 (Gibco, USA) containing 10% fetal bovine serum (Gibco), at 28 °C. Cells were subcultured at intervals of 3 days. Twenty-four hours before