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TAK1-binding proteins (TAB1 and TAB2) in grass carp (*Ctenopharyngodon idella*): Identification, characterization, and expression analysis after infection with *Ichthyophthirius multifiliis*

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ABSTRACT

Transforming growth factor-β activated kinase-1 (TAK1) is a key regulatory molecule in toll-like receptor (TLR), interleukin-1 (IL-1), and tumor necrosis factor (TNF) signaling pathways. The activation of TAK1 is specifically regulated by two TAK1-binding proteins, TAB1 and TAB2. However, the roles of TAB1 and TAB2 in fish have not been reported to date. In the present study, TAB1 (CiTAB1) and TAB2 (CiTAB2) in grass carp (*Ctenopharyngodon idella*) were identified and characterized, and their expression profiles were analyzed after fish were infected with the pathogenic ciliate *Ichthyophthirius multifiliis*. The full-length CiTAB1 cDNA is 1949 bp long with an open reading frame (ORF) of 1497 bp that encodes a putative protein of 498 amino acids containing a typical PP2Cc domain. The full-length CiTAB2 cDNA is 2967 bp long and contains an ORF of 2178 bp encoding a putative protein of 725 amino acids. Protein structure analysis revealed that CiTAB2 consists of three main structural domains: an N-terminal CUE domain, a coiled-coil domain, and a C-terminal ZnF domain. Multiple sequence alignment showed that CiTAB1 and CiTAB2 share high sequence identity with other known TAB1 and TAB2 proteins, and several conserved phosphorylation sites and an O-GlcNAc site were deduced in CiTAB1. Phylogenetic tree analysis demonstrated that CiTAB1 and CiTAB2 have the closest evolutionary relationship with TAB1 and TAB2 of *Danio rerio*, respectively. CiTAB1 and CiTAB2 were both widely expressed in all examined tissues with the highest levels in the heart and liver, respectively. After infection with *I. multifiliis*, the expressions of CiTAB1 and CiTAB2 were both significantly up-regulated in all tested tissues at most time points, which indicates that these proteins may be involved in the host immune response against *I. multifiliis* infection.

1. Introduction

Transforming growth factor-β activated kinase-1 (TAK1), also known as mitogen-activated protein kinase kinase kinase 7 (MAP3K7), is a serine/threonine kinase in the mitogen-activated protein kinase (MAPK) family. It functions as a key regulatory molecule in toll-like receptor (TLR), interleukin-1 (IL-1), and tumor necrosis factor (TNF) signaling pathways [1]. Two TAK1-binding proteins, TAB1 and TAB2, which tightly regulate the activation of TAK1, were originally identified via the yeast two-hybrid screening using TAK1 protein as bait [2,3].

In recent years, significant progress has been made in elucidating the essential roles played by mammalian TAB1 and TAB2 [4,5]. Like the knock-out of TAK1 in mice, the knock-out of either TAB1 or TAB2 resulted in embryonic lethality in mice [6–8]. TAB1 interacts constitutively with the N-terminal of TAK1 and directly induces TAK1 kinase activity [2], whereas TAB2 acts as an adaptor that links the C-terminal of TAK1 to the upstream TRAF6 or TRAF2 and thereby mediates the activation of TAK1 but does not directly activate TAK1 [3,9]. In the TLR signaling pathway, following activation by their cognate ligands, all mammalian TLRs (with the

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exception of TLR3), can in turn recruit MyD88, IRAK4, and IRAK1 [10]. Subsequently, TAB2 links polyubiquitinated TRAF6 and TAK1-TAB1 to form the TRAF6–TAK1–TAB1–TAB2 complex [3]. TAB1 then activates TAK1 to trigger activation of the transcription factors NF-κB and AP-1, which cooperatively modulate the expression of proinflammatory cytokines in response to pathogen infection [2,5]. In addition to TAK1, TAB1 can also directly bind to p38α MAPK and activate its autophosphorylation to participate in the feedback regulation of TAK1 activity [11–13]. TAB2 also has several other novel functions, such as promoting TRAF6 ubiquitination and facilitating the assembly of TRAF6 with IKK [14]. However, the mechanisms by which TAB1 and TAB2 act are not yet completely clear. In particular, their roles in fish have never been reported to date.

Ichthyophthirius multifiliis is a highly pathogenic ciliate that infects almost all freshwater fish species and causes ichthyophthiriosis, or "white spot disease" [15,16]. Moreover, *I. multifiliis* infection can enhance invasion by other pathogens, thus causing increased mortality [17,18]. Because *I. multifiliis* infection is easily controlled under laboratory conditions, it has been used as a model to study the immune response of parasitized fish in many laboratories, including our lab [19–23]. Grass carp (*Ctenopharyngodon idella*) is one of the most important aquaculture species in China, and it accounts for about 20% of the total annual output of freshwater cultured fish. However, this species has suffered high mortality in recent years due to diseases, especially infection by *I. multifiliis*.

Results of our recent study indicated that grass carp TRAF6 and TAK1 may play crucial roles in the immune response against *I. multifiliis* infection [22]. In order to better understand the functions of TAB1 and TAB2 in immune responses and to compare the biological roles of the two molecules in fish and mammals, we identified the cDNA sequences of TAB1 (CiTAB1) and TAB2 (CiTAB2) in grass carp, described their expression pattern in different tissues, and analyzed their expression profiles after infection with *I. multifiliis*. To our knowledge, this is the first report describing the tissue distribution and the expression pattern induced by pathogen infection of both genes in fish.

2. Materials and methods

2.1. Fish and parasite

Healthy grass carp weighing approximately 50 g were purchased from the aquaculture base at Pearl River Fisheries Research Institute, Guangzhou, China. Fish were acclimated in a recirculating water tank system at 23 ± 1 °C for 2 weeks preceding the experiment. They were fed twice daily using a commercial pellet diet.

In a laboratory system, *I. multifiliis* was maintained by serial transmission on channel catfish (*Ictalurus punctatus*) with an average body weight of 45 g. Live theronts for the infection experiment were collected according to previously described procedure [23].

2.2. Homologous cloning of partial CiTAB1 and CiTAB2 cDNA sequences

The homologous fragment of CiTAB1 cDNA was cloned using degenerate primers TAB1.F3/TAB1.R3 (Table 1), which were designed based on multiple sequence alignment of some known TAB1 genes, including those from *Danio rerio*, *Oreochromis niloticus*, *Mus musculus*, *Cricetulus griseus*, and *Sus scrofa* (GenBank Accession Nos. XM_002662240, XM_005464854, NM_025609, XM_003509459, and NM_001244067, respectively). Likewise, degenerate primers TAB2.F2/TAB2.R4 (Table 1) for CiTAB2 were designed based on TAB2 genes from *O. niloticus*, *Anolis carolinensis*, *Gallus gallus*, *C. griseus*, *M. musculus*, and *Ornithorhynchus anatinus* (GenBank Accession Nos. XM_003453021, XM_003224691, XM_419660, XM_003498946, NM_138667, and XM_003428333, respectively).

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)	Application
TAB1.F3	GGCTTCYTGBTGCTGATGTC	Homologous
TAB1.R3	GAGCTGGAGCTGWSGCTCTG	cloning
TAB1.3F1	ACGGATCACTCACTCCCACACAAGG	3′RACE
TAB1.5R1	GCTGGTCTTACTGGTGCTCTGATGGT	5′RACE
TAB1.5R2	CAGAGCCAACTCCGCTGCTACCAT	
TAB2.F2	ACBCCYACDTCTTTGCACATACA	Homologous
TAB2.R4	CYGGGRTTAAARTGTGGTCCT	cloning
TAB2.3F1	TGCTGGTCTGCGGGCTTTAGGAA	3′RACE
TAB2.5R1	GGCGGTTTATTGGGGGAGACAGTGATTT	5′RACE
TAB2.5R2	AGATGGAGTTTGGACTGTTCAGACCCGA	
3′-CDS	AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN	3′RACE
5′-CDS	$(T)_{25}$VN	5′RACE
SMARTer IIA oligo	AAGCAGTGGTATCAACGCAGAGTACXXXXX	5′RACE
UPM	Long: CTAATACGACTCACTATAGGGCAAGC AGTGGTATCAACGCAGAGT Short: CTAATACGACTCACTATAGGGC	RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	Nested RACE
rTAB1.F	CACAACCGACAATGAAGAT	Real-time PCR
rTAB1.R	GGAGATGATTGGCTTATGC	
rTAB2.F	TGCCTATCAGTTCACCTAC	Real-time PCR
rTAB2.R	GTTGAGATGTTCTGGATGTTAT	
rβ-actin.F	TCTGCTATGTGGCTCTTG	Real-time PCR
rβ-actin.R	ACCTGAACCTCTCATTGC	

Note: Y = C/T; S = C/G; R = A/G; W = A/T; B = C/G/T; *D* = A/G/T; V = A/G/C; N = A/G/C/T.

Total RNA was isolated from the spleen of grass carp using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. Subsequently, any genomic DNA contamination was eliminated through treatment using RNase-free DNase I (Fermentas, USA) at 37 °C for 30 min. The treated RNA (1 μg) was reverse transcribed into cDNA using ReverTra Ace-α-reverse transcriptase (TOYOBO, Japan), which was used as a template for amplification.

The PCR program was as follows: 94 °C for 2 min followed by 35 cycles at 94 °C for 45 s, 52 °C for 45 s, 72 °C for 1.5 min, and 72 °C for 7 min. The PCR products were gel purified, ligated into the pMD18-T vector (TaKaRa, Japan), and transformed into the competent cells (*Escherichia coli* DH5α). Three positive recombinant clones for each gene identified by colony PCR were sequenced. Two cDNA sequences (506 bp and 1418 bp) were obtained, which revealed high sequence identity to TAB1 and TAB2 genes of other fish species, respectively.

2.3. Cloning of the full-length cDNA of CiTAB1 and CiTAB2

Based on the partial sequences of CiTAB1 and CiTAB2 obtained above, the 3′ and 5′ ends were amplified by rapid amplification of cDNA ends (RACE) procedures, which were performed using the SMARTer™ RACE cDNA amplification kit (Clontech, USA) according to the manufacturer's protocol. In brief, the first strand cDNA was synthesized from 1 μg of total spleen RNA using the 5′- CDS Primer A and SMARTer IIA oligo for 5′ RACE and the 3′- CDS Primer A for 3′ RACE (Table 1), respectively. For CiTAB1, primers TAB1.3F1/UPM (Table 1) were used to clone the 3′ unknown region, and primers TAB1.5R1/UPM, TAB1.5R2/NUP (Table 1) were used to clone the 5′ unknown region for the primary PCR and the nested PCR, respectively. Similarly, for CiTAB2, the 3′ and 5′ unknown regions were obtained using primers TAB2.3F1/UPM, TAB2.5R1/UPM, and TAB2.5R2/NUP (Table 1). The PCR program consisted of 1 cycle at 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. The product of the primary PCR was diluted 50