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Phylogenetically conserved TAK1 participates in *Branchiostoma belcheri* innate immune response to LPS stimulus

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ABSTRACT

Transforming growth factor-β activated kinase-1 (TAK1) is an important member of the mitogen-activated protein kinase kinase kinase (MAP3K) family, which plays an important role in animal innate immune response. However, the *TAK1* gene has yet not been reported in amphioxus to date. Here, we have identified and characterized a *TAK1* gene from amphioxus (*Branchiostoma belcheri*) (named as *AmphiTAK1*) with the full-length cDNA of 3479 bp, including an ORF sequence of 1905 bp, a 5′ UTR of 394 bp and a 3′ UTR of 1180 bp. Moreover, the predicted AmphiTAK1 protein contains STKc_TAK1 domain, TAB1 and TAB2/3 binding domain which are conserved among chordate, and phylogenetic analysis also shows that the AmphiTAK1 is located at the bottom of the chordate, revealing *AmphiTAK1* as a new member of the *TAK1* gene family. The further qRT-PCR analysis has shown that *AmphiTAK1* is widely expressed in six investigated tissues (gonad, gill, hepatic cecum, intestine, muscle and notochord) of *Branchiostoma belcheri*, with high expression in notochord and gonad, moderate in gill and hepatic cecum. Notably, the expression level of *AmphiTAK1* is significantly up-regulated after LPS stimulation. Specially, we also find that AmphiTAK1 protein can interact with AmphiTAB1 by immunoprecipitation assay. These findings reveal that AmphiTAK1 might interact with AmphiTAB1 to involve in innate immune response of *Branchiostoma belcheri*. Taken together, our present works provide a new insight into evolution and innate immune response mechanism of *AmphiTAK1* gene in *Branchiostoma belcheri*.

1. Introduction

Mitogen-activated protein kinases (MAPKs) are highly conserved in eukaryotic organisms, which can regulate a variety of cellular activities, including inflammation, apoptosis, proliferation, development, differentiation and other metabolic activities [1–5]. The MAPK signaling cascades mainly include these pathways of ERK1/2, JNK, p38 MAPK and ERK5/BMK1 [1,6]. The activation of MAPK signaling is a typically hierarchical three-tiered core, namely MAP3Ks, as key activators, firstly activate MAP2Ks, and then MAP2Ks activate MAPKs to further promote the expressions of certain downstream signaling molecules, such as transcription factors, cytoskeletal elements, membrane transporters, nuclear pore proteins and other protein kinases, to perform a variety of biological functions [7,8].

Especially, in vertebrates, MAP3K family has at least 20 different members, and they can selectively phosphorylate and activate different MAP2Ks to further tightly control the activation of specific MAPKs in response to distinctly extracellular stimulus [9]. These identified MAP3K proteins mainly include the following six categories: mitogen-activated protein/ERK kinase kinases(MEKKs), mixed lineage kinases (MLKs), Rafs, thousand and one amino acid TAO/tumor progression locus 2 (Tpl2), as well as MOS and TGF-bactivated kinase 1 (TAK1) [9]. At present, many works have been widely focused on the functions and evolutionary mechanisms of MAP3K family members.

The TAK1 is an important member of MAP3K family, which is originally discovered in the TGF-beta signaling pathway [8]. TAK1, as a serine/threonine kinase, is an important signaling molecule involved in various physiological processes including tumorigenesis, vascular development, neural fold morphogenesis, innate and adaptive immune responses [10–12]. TAK1, as a very pivotal activator of NF-κB and MAPK signaling, can phosphorylate downstream IB kinase (IKK) complex, ERK, JNK, p38 MAPK to activate NF-κB and MAPK signaling pathways to participate in immune responses [13]. TAK1 can form multimeric protein complexes with its TAB1 and TAB2/3 partners,

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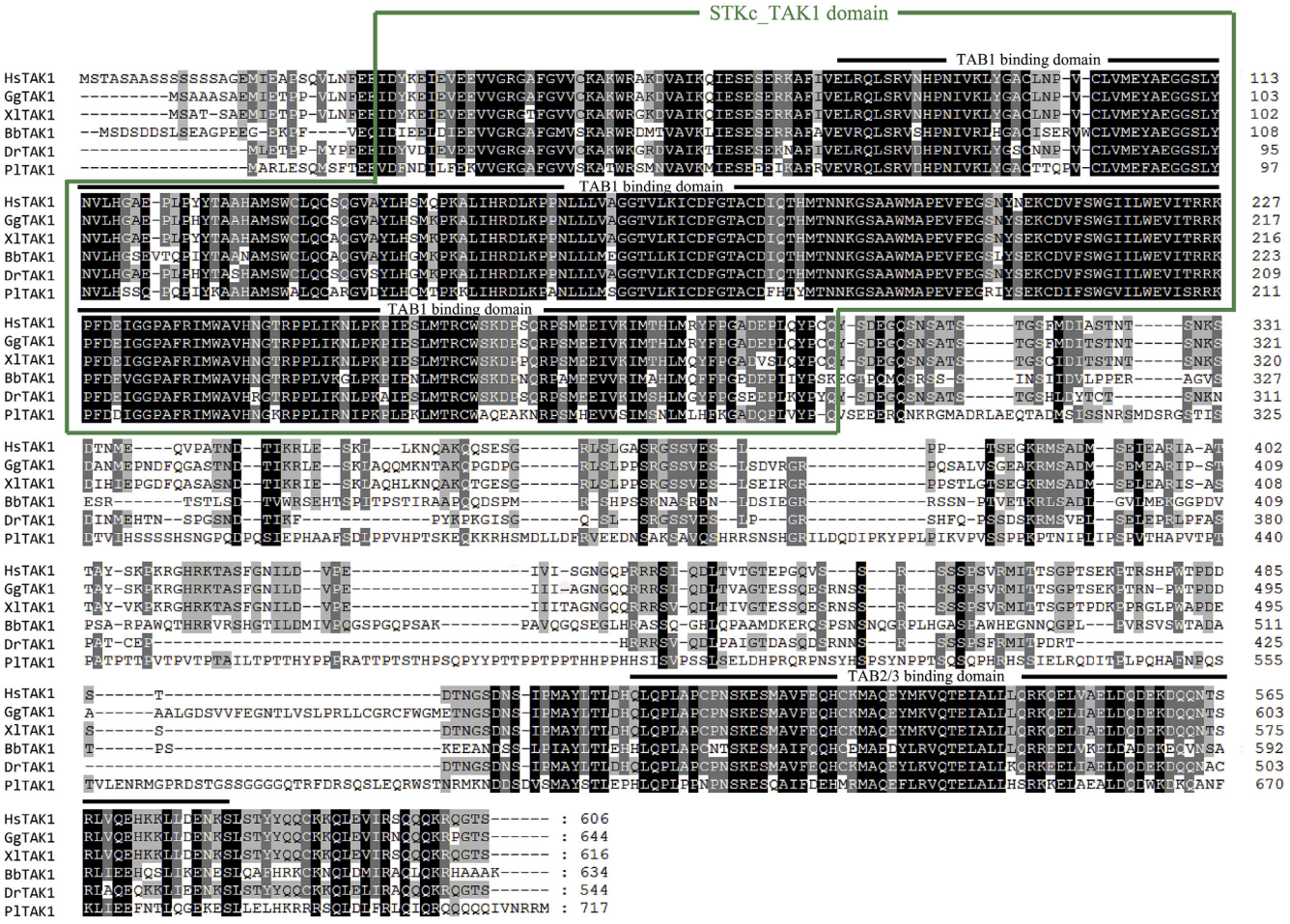

Fig. 1. The sequence alignment of TAK1 proteins. The STKc_TAK1 domain is in the green box. The TAB1 binding domain and TAB2/3 binding domain are located below the black line. The figure includes the following species: HsTAK1, *Homo sapiens* TAK1 (GenBank,AAV38461.1); GgTAK1, *Gallus gallus* TAK1 (GenBank, XP_015140163.1); XlTAK1, *Xenopus laevis* TAK1 (GenBank,NP_001084359.1); BbTAK1, *Branchiostoma belcheri* TAK1; DrTAK1, *Danio rerio* TAK1 (GenBank, NP_001018586.1); PlTAK1, *Paracentrotus lividus* TAK1 (GenBank, ABF82443.1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

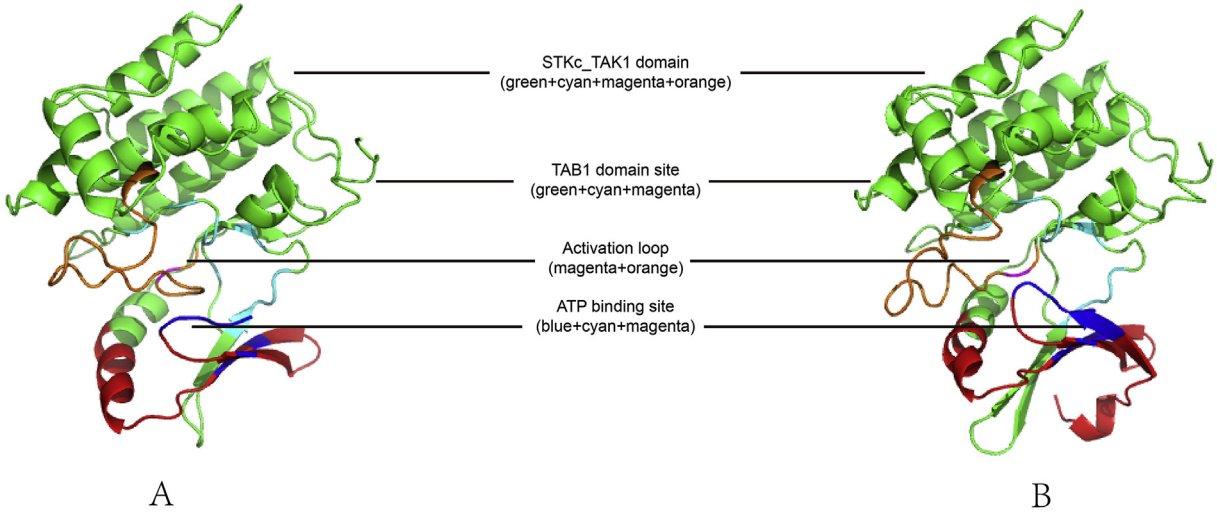

Fig. 2. The three dimensional structure of STKc_TAK1 domain of *Homo sapiens* (A) and *Branchiostoma belcheri* (B). The STKc_TAK1 domain include: green, cyan, magenta and orange; The TAB1 binding domain include: green, cyan and magenta; The activation loop include: magenta and orange. The ATP binding site include: blue, cyan and magenta. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

Table 1
Homology analysis of AmphiTAK1 protein with other known TAK1 proteins.

Species	Similarity(%)	Identify(%)
Homo sapiens	71.1	52.2
Bos taurus	69.7	51.4
Mus musculus	70.0	51.9
Philosophers sinicus	71.6	51.9
Anas platyrhynchos	69.3	50.5
Taeniopygia guttata	70.5	51.6
Ophiophagus hannah	69.2	52.2
Gekko japonicus	70.2	52.8
Chrysemys picta bellii	68.1	50.7
Xenopus laevis	67.9	51.9
Epinephelus coioides	69.8	52.4
Oncorhynchus kisutch	71.5	54.4
Tribolium castaneum	54.7	37.9
Apis mellifera	58.2	40.3

namely TAB1 and TAB2/3 can, respectively, interact with the N-terminal kinase domain and C-terminal regulatory domain of TAK1 to activate NF-kB and JNK signaling [14–19]. Taken together, these studies reveal the key roles of TAK1 in regulating NF-kB and JNK signaling for cell survival as well as innate and adaptive immune responses.

At present, the functions of TAK1 have been widely studied in many species. However, the study on TAK1 of *Branchiostoma belcheri* has yet not been reported. The *Branchiostoma belcheri* belonging to the genus of the chordate represents a transitional species from invertebrates to vertebrates, thus it is an important species to study the origin and evolution of the immune system of chordate [20,21]. Therefore, to further reveal the functional role of the *TAK1* gene of amphioxus (*Branchiostoma belcheri*), we here have cloned the full length of the

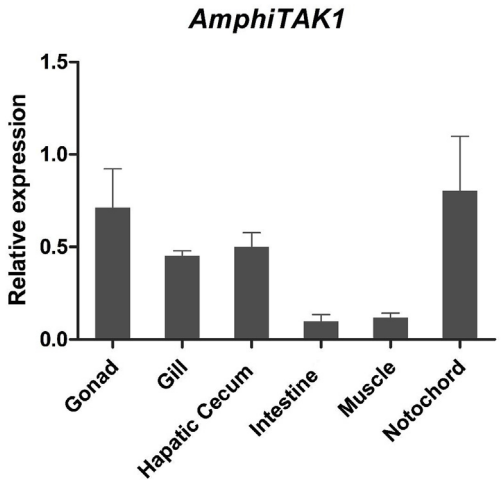

Fig. 4. *AmphiTAK1* gene expression in different tissues. Quantitative real-time PCR was performed with RNA samples from gonad, gill, hapatic cecum, intestine, muscle and notochord of *Branchiostoma belcheri*. All samples were run in three duplications. The results were shown in terms of relative mRNA level as mean ± SE (n = 3). The β-actin gene of amphioxus was used as internal control for calibrating the cDNA template of *Branchiostoma belcheri*.

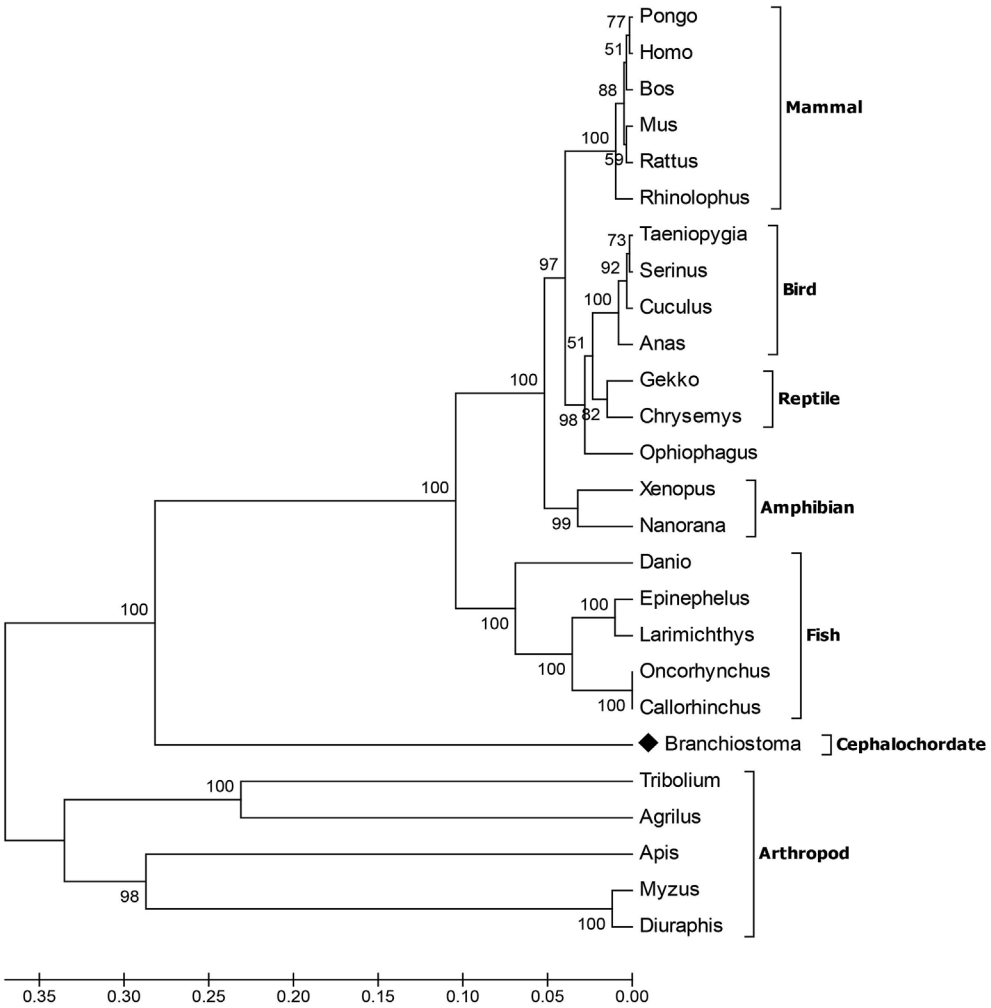

Fig. 3. The phylogenetic tree of TAK1 protein family. The phylogenetic tree was constructed (maximum-likelihood algorithm) using MEGA6. The reliability of results was verified from 1000 bootstrap repetitions. *Pongo abelii* TAK1 (GenBank, NP_001124617.1); *Homo sapiens* TAK1 (GenBank, AAV38461.1); *Bos taurus* TAK1 (GenBank, NP_001075064.1); *Mus musculus* TAK1(GenBank, NP_033342.1); *Rattus norvegicus* TAK1 (GenBank, NP_001101390.2); *Rhinolophus sinicus* TAK1 (GenBank, XP_019598832.1); *Taeniopygia guttata* TAK1 (GenBank, XP_005518268.1); *Serinus canaria* TAK1 (GenBank, XP_009091818.1); *Anas platyrhynchos* TAK1 (GenBank, EOB06607.1); *Gekko japonicus* TAK1 (GenBank, XP_015271965.1); *Chrysemys picta bellii* TAK1 (GenBank, XP_005299791.1); *Ophiophagus hannah* TAK1 (GenBank, ETE70582.1); *Xenopus laevis* TAK1 (GenBank, NP_001084359.1); *Nanorana parkeri* TAK1 (GenBank, XP_018427552.1); *Danio rerio* TAK1 (GenBank, NP_001018586.1); *Epinephelus coioides* TAK1 (GenBank, AGQ48129.1); *Larimichthys crocea* TAK1 (GenBank, XP_010735617.1); *Oncorhynchus kisutch* TAK1 (GenBank, XP_020310256.1); *Callorhinchus milii* TAK1 (GenBank, XP_007891110.1); *Branchiostoma belcheri* TAK1 (GenBank, XP_019647761.1); *Tribolium castaneum* TAK1 (GenBank, XP_968547.1); *Agrilus planipennis* TAK1 (GenBank, XP_018333073.1); *Myzus persicae* TAK1 (GenBank, XP_022182763.1); *Diuraphis noxia* TAK1 (GenBank, XP_015374221.1).

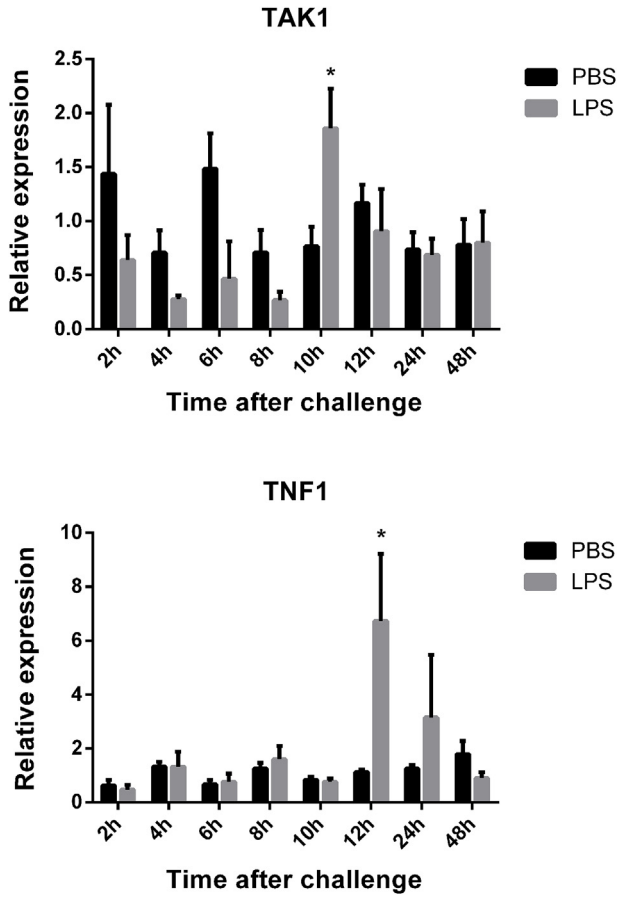

Fig. 5. Expression analysis of *AmphiTAK1* and *AmphiTNF1* after LPS stimulation. Mature amphioxus adults (*Branchiostoma belcheri*) after LPS stimulation at different time points were used as different experimental groups. Mature amphioxus adults (*Branchiostoma belcheri*) after PBS stimulation at different time points were used as corresponding control groups. All samples were run in three duplications. The results were shown in terms of relative mRNA level as mean ± SE (n ≥ 3). The *β-actin* gene of *Branchiostoma belcheri* was used as internal control for calibrating calibrating the cDNA template of *Branchiostoma belcheri*.

AmphiTAK1, and analyzed the gene structure, protein homology, and the phylogenetic relationship of the *AmphiTAK1*. Especially, we have analyzed the expression level of *AmphiTAK1* in various tissues of amphioxus, and the temporal expression change of *AmphiTAK1* after LPS stimulation. Finally, we have found that AmphiTAK1 may interact with AmphiTAB1 by co-immunoprecipitation assay. Our findings have indicated that *AmphiTAK1* is a new member of the *TAK1* gene family, and AmphiTAK1 may interact with AmphiTAB1 to participate in innate immune response of *Branchiostoma belcheri*.

2. Materials and methods

2.1. Amphioxus and cell culture

Mature amphioxus adults (*Branchiostoma belcheri*) were captured in Zhanjiang (Guangdong province, China) and cultured in artificial seawater (24°C–25 °C) circulating with an air-pumped, feeding with chlorella at a fixed time daily and the lighting conditions is simulated by a fluorescent lampand.

HEK293 cells were cultured in DMEM medium with 10% FCS, and 1% Penicillin-Streptomycin Solution were incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Immune stimulation of amphioxus with LPS

These adult amphioxus were exposed to sterilized seawater containing PBS or LPS (10 μg/ml; dissolved in PBS solution), referring to the experimental methods of Guo et al. [22]. Then these samples were collected at different time points (2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h and 48 h) and rapidly frozen in liquid nitrogen for extracting the total RNA.

2.3. Cloning of the full-length sequence of AmphiTAK1 cDNA by PCR

The total RNA from *Branchiostoma belcheri* was extracted by using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The first strand of *Branchiostoma belcheri* cDNA was synthesised using Moloney Murine Leukemia Virus reverse transcriptase (M-MLV; TaKaRa, Dalian, China). A pair of primers (F: 5′-AAACCTCCGAAATGAGCGACT-3'; R: 5′-GGTTGTGTCCGTTACTTG GCT-3′) were designed according to the *TAK1* gene fragment of *Branchiostoma belcheri*, and the cDNA conserved segment of *Brachiosaurus belcheri* was cloned by using the pair of primers. In order to obtain the full length cDNA of *AmphiTAK1*, both 5′RACE and 3′RACE were performed using SMARTer RACE cDNA Amplification Kit (Clontech, CA, USA) and First Choice RLM-RACE Kit (Ambion, Austin, TX, USA) according to the product manual, respectively. Following the manufacturer's instructions, a pair of primers (F: 5′-CTCTTCTTTGCTG GGGGTGGCGTCT-3'; R: 5′-TATGAGTGCCTTGGGTTTCATGCCGT-3′) were designed for 5′RACE, and another pair of primers (F: 5′-CAGTG AAGTGACCCAGCCCATCT-3'; R: 5′-AGACGCCACCCCCAGCAAAG-3′) were designed for 3′RACE. Finally, a pair of primers (F: 5′-TCTCGCC GCATCCTAAGTC-3'; R: 5′-CAAGCAAACACGAAACCAAT-3′) were designed to amplify the full-length cDNA sequence of *Branchiostoma belcheri* to ensure the accuracy of the *AmphiTAK1* cDNA sequence. Then the full-length cDNA sequence of *Branchiostoma belcheri* was connected into the pMD® 19-T Simple Vector by gene cloning technology and sequenced.

2.4. Sequence alignment, structural analysis and phylogenetic analysis of AmphiTAK1

In order to perform TAK1 protein sequence alignment, we entered the full length of the *TAK1* cDNA of *Branchiostoma belcheri* into the Open Reading Frame Finder tool (https://www.ncbi.nlm.nih.gov/orffinder/) to obtain the TAK1 protein sequence of *Branchiostoma belcheri*. Furthermore, we downloaded TAK1 protein sequences of other representative species from JGI database (http://genome.jgi-psf.org) and NCBI database (https://www.ncbi.nlm.nih.gov/), respectively [23]. Subsequently, we predicted TAK1 protein functional domain of each species by using NCBI Conserved Domain Search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). We used MatGAT program to calculate the similarity and identity among different TAK1 proteins with default parameters [24]. Multiple alignments of TAK1 proteins were performed using the GENDOC program. We constructed three-dimensional structure of TAK1 proteins using SWISS-MODEL prediction software (https://www.swissmodel.expasy.org/), and showed the structure of TAK1 protein using the Pymol-0_97-bin-win32 program. Finally, we constructed the phylogenetic tree (maximum-likelihood algorithm) using MEGA6 software, and the reliability of result was verified from 1000 bootstrap repetitions [25].

2.5. Quantitative real-time PCR of AmphiTAK1

To explore the spatial expression of *AmphiTAK1* in various tissues of *Branchiostoma belcheri*, total RNA was extracted from the gonad, gill, hepatic cecum, intestine, muscle and notochord of *Branchiostoma belcheri*. In order to explore the change in mRNA expression level of *TAK1* gene at different times after LPS stimulation, we extracted total RNA at different time points after LPS stimulation. All RNA were extracted

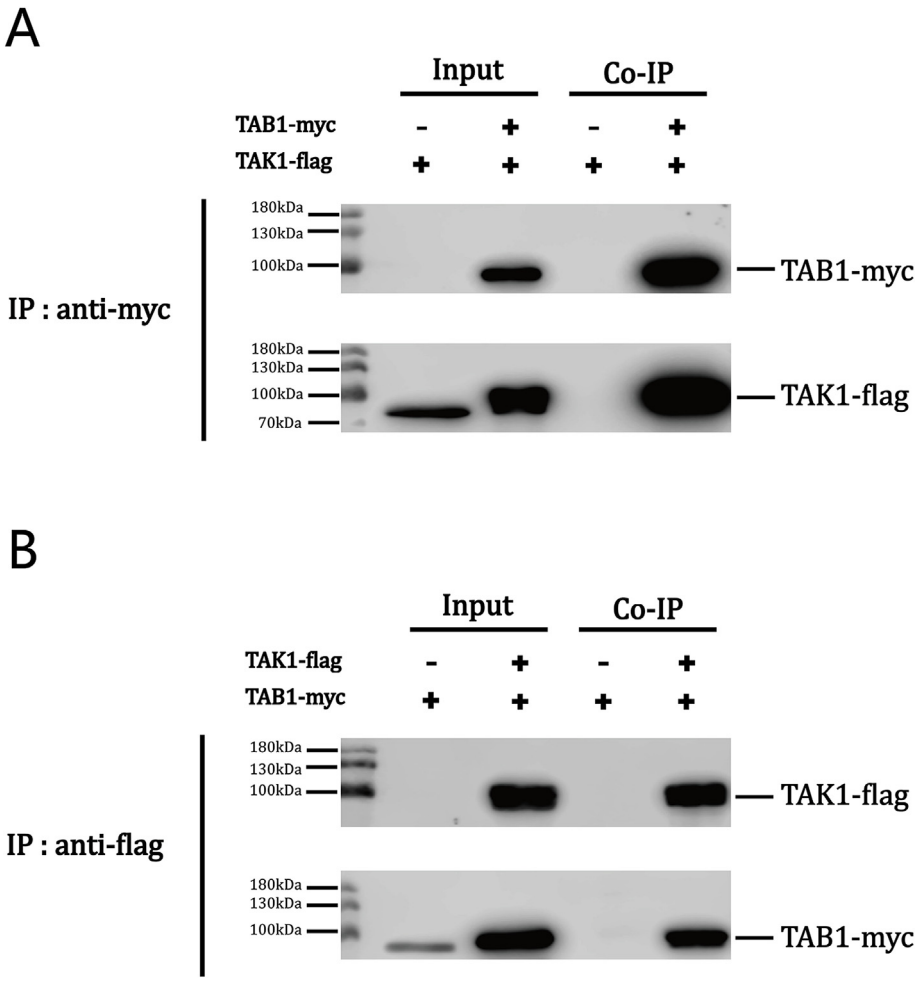

Fig. 6. The interaction of AmphiTAK1 with AmphiTAB1 by co-immunoprecipitation. Co-IP assay result (A) showed that the AmphiTAK1 can be co-precipitated by AmphiTAB1 through using Anti-Myc immunomagnetic bead. Co-IP assay result (B) showed that the AmphiTAB1 can be co-precipitated by AmphiTAK1 through using ANTI-FLAG M2 Affinity Gel.

using Trizol reagent (Invitrogen, Carlsbad, USA) and all cDNAs were synthesised using the PrimeScript RT kit (TaKaRa, Dalian, China). Two gene-specific quantitative primers of *AmphiTAK1* gene (F:5′-GTGGTG CTTAGTGATGGAGTATGC-3'; R:5′-CTGTGTATGAGTGCCTTGGGT TTC-3′) were designed to amplify a product of 171 bp using Beacon Designer 7.0 software, and the *β-actin* gene of *Branchiostoma belcheri* (F: 5′-GCCTCCCTGTCCACCTTCC-3'; R:5′-AACTTGCCATCCTTAGCCA CTG-3′) was used as internal control for calibrating the cDNA template of *Branchiostoma belcheri*. In addition, to further clarify that the *AmphiTAK1* gene is involved in the pro-inflammatory response, we designed gene-specific quantitative primers of *AmphiTNF1* (F:5′-GCCG CTAACTGGATACTACTAC-3'; R:5′-CACCGTCTTGGGAGGAAAC-3′) with a product of 182 bp to check the expression pattern of proinflammatory cytokines. The reaction system volume of RT-PCR is 15ul in total, including 5.4 μl of double-distilled water, 0.3 μl of each primer and 7.5 μl of 2 × SYBR Green Real time PCR MasterMix, 1.5 μl of cDNA. The PCR reaction conditions were performed: 95 °C for 10 min followed by 40 cycles of 95 °C for 10s and 55 °C for 30s. Real-time PCR reaction was executed in StepOnePlus™ Real-Time PCR System (ABI, Switzerland). All samples were run in three duplications, and the results were shown in terms of relative mRNA level as mean ± SE (n ≥ 3). Then the result was carried out the *t*-test with statistical significance for $P < 0.05$ by statistical software (GraphPad Prism 5.0).

2.6. Co-immunoprecipitation about AmphiTAK1 and AmphiTAB1

In order to detect the interaction between AmphiTAK1 and AmphiTAB1 in *Branchiostoma belcheri*, we obtained the *AmphiTAB1* ORF sequence with the myc-tag at the 5′ end and the *AmphiTAK1* ORF sequence by PCR. We connected the *AmphiTAK1* and the *AmphiTAB1* sequence to the commercial plasmid vectors p3xFLAG and pcDNA3.1 (+), respectively. Then we transfected the constructed plasmid into HEK293 cells and cultured for 48 h in a humidified atmosphere of 5% CO_2 at 37 °C. After 48 h, the cells were lysed with a Co-IP lysate (Beyotime) and treated with Anti-Myc immunomagnetic bead (Bimake) or ANTI-FLAG M2 Affinity Gel (Sigma) following the manufacturer's protocol. One tenth of the volume of the cell lysate was collected as the input control group and the bead or Affinity Gel was incubated with the remaining cell lysate at 4 °C overnight. The Anti-Myc immunomagnetic bead or ANTI-FLAG M2 Affinity Gel was washed three times with washing buffer the next day. Then the Anti-Myc immunomagnetic bead or ANTI-FLAG M2 Affinity Gel was added to the 1 × loading buffer and boiled for 10 min. Finally, the obtained protein samples were detected by Western Blot.

3. Results

3.1. Cloning and characterization of the AmphiTAK1

Taking advantage of the database of *Branchiostoma belcheri* genome

(http://genome.bucm.edu.cn/lancelet/index.php), a 1927 bp fragment of the *AmphiTAK1* gene was obtained by RT-PCR. According to this 1927 bp fragment, we obtained a 866 bp and a 1582 bp fragment by 5′-RACE and 3′-RACE, respectively. Three fragments were assembled to obtain the full sequence of the *AmphiTAK1* cDNA. The complete *AmphiTAK1* cDNA was verified by end-to-end PCR assay. The results showed that the full length of the *AmphiTAK1* cDNA is 3479 bp, with the 5′-UTR region of 394 bp, the 3′-UTR of 1180 bp, and the ORF of 1905 bp that encoding a polypeptide of 634 amino acid (Fig. S1A, Fig. S1B). The predicted AmphiTAK1 protein contains a conservative STKc_TAK1 domain (Catalytic domain of the Serine/Threonine Kinase) at 31-303aa, and a TAB1 binding domain at 77-303aa (Fig. S1B), as well as a TAB2/3 binding domain at 479-553aa of the C-terminal (Fig. S1B). The *AmphiTAK1* full-length cDNA sequence and the predicted protein sequence had been uploaded to GenBank (ID: MK818446).

3.2. Alignment and phylogenetic analysis of AmphiTAK1

The gene structure of *AmphiTAK1* is composed of eighteen exons interrupted by seventeen introns, and the phase and structure of *AmphiTAK1* is different from other five species (Fig. S2). Interestingly, the STK_c TAK1 domain (31-303aa), TAB1 binding domain (77-303aa), and TAB2/3 binding domain (533-607a) in AmphiTAK1 are conserved among the six species (Fig. 1), the AmphiTAK1 protein also includes the conserved ATP binding site (35-171aa) and activation loop (170-190aa) by using the NCBI Conserved Domain Search Tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Additionally, the three-dimensional structures of *Homo sapiens* TAK1 protein and *Branchiostoma belcheri* TAK1 protein are also highly similar (Fig. 2A, Fig. 2B). The similarity of AmphiTAK1 sequence with other species is about 54.7%–71.6%, and the identity is about 37.9%–54.4% (Table 1). Specially, the phylogenetic tree also showed that AmphiTAK1 is located between vertebrates and invertebrates, and AmphiTAK1 clusters with the vertebrate TAK1 protein (Fig. 3). Taken together, these results indicate that *AmphiTAK1* is a member of the *TAK1* gene family.

3.3. Expression levels of AmphiTAK1 in different tissues

In order to profile the mRNA spatial distribution of *TAK1* gene in various tissues of *Branchiostoma belcheri*, we investigated the expression levels of *AmphiTAK1* in gonad, gill, hepatic cecum, intestine, muscle and notochord by quantitative RT-PCR. The results demonstrated that *AmphiTAK1* was expressed in six various tissues of *Branchiostoma belcheri*, and the expression level of *AmphiTAK1* was high in notochord and gonad, moderate in gill and hepatic cecum, and low in intestine and muscle (Fig. 4).

3.4. Expression level of AmphiTAK1 after LPS stimulation

In order to explore the potential function role of *AmphiTAK1* in innate immune response, we further used qRT-PCR to detect the expression levels of *AmphiTAK1* at different time points (2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h, 48 h) after LPS stimulation (Fig. 5). The results showed that the expression level of *AmphiTAK1* was significantly increased at 10 h after LPS stimulation, which was 2.43-fold of the control group ($P < 0.05$). Then the expression of *AmphiTAK1* gradually returned to normal level. Intriguingly, the expression level of proinflammatory cytokines *AmphiTNF1* was also significantly increased at 12 h compared to the control group (5.95-fold; $P < 0.05$). Taken together, these above results revealed that *AmphiTAK1* might participate in the innate immune response of *Branchiostoma belcheri*.

3.5. Protein interaction between AmphiTAK1 and AmphiTAB1

To further reveal if AmphiTAK1 may interact with AmphiTAB1 in *Branchiostoma belcheri*, we transfected or cotransfected the *AmphiTAK1* and *AmphiTAB1* gene into HEK293 cells. At 48 h after transfection, we used Anti-Myc immunomagnetic bead and Anti-Flag affinity gel for co-immunoprecipitation experiments, respectively (Fig. 6). We pull down TAB1 protein using Anti-Myc immunomagnetic bead, and verified the presence of TAK1 protein using Western Blot. The result indicated that the TAK1 protein might be pulled down by TAB1 protein. Similarly, TAB1 protein could also be pulled down using Anti-Flag affinity gel. The result indicated that TAK1 protein could interact with TAB1 protein regardless of using Anti-Myc immunomagnetic bead or Anti-Flag affinity gel. These above results seem to reveal that AmphiTAK1 interacts with AmphiTAB1 to participate in innate immune response of *Brachiosaurus belcheri*.

4. Discussion

In this study, we have cloned and characterized a new *AmphiTAK1* gene from amphioxus, which is a member of the *TAK1* gene family. Especially, the AmphiTAK1 protein contains STKc_TAK1 domain, TAB1 binding domain, TAB2/3 binding domain, ATP binding site and activation loop (Figs. 1 and 2), which is highly conserved compared to other species, suggesting that *AmphiTAK1* could exert similar biological functions with vertebrate *TAK1* gene.

The *AmphiTAK1* gene could be expressed in gonad, gill, hepatic cecum, intestine, muscle and notochord of amphioxus (Fig. 4), implying that the *AmphiTAK1* may play multiple biological function roles. The highest *AmphiTAK1* expression level in notochord may be related to the tissue-specific functions of *AmphiTAK1*. The second-highest *AmphiTAK1* expression level in gonad, suggesting a potential role in gametogenesis and maintaining the homeostasis of the gonad [26,27]. The relatively high *AmphiTAK1* expression levels in gill and hepatic cecum may indicate that they paly key roles in response to the external environment or aquatic microorganism stimulus. The hepatic cecum of amphioxus is a precursor of vertebrate liver, which should have similar functions with the vertebrate's liver, thus *AmphiTAK1* may participate in the acute phase response with similar to vertebrates [28,29]. Moreover, many studies have shown that the expression of the *TAK1* gene is closely related to the inflammatory response [30–33], suggesting that the high expression of *AmphiTAK1* may be also associated with inflammatory response. Collectively, our present works reveal that *AmphiTAK1* has similarly biological functions with vertebrate *TAK1* gene.

LPS can effectively activate innate immune pathways [34]. Therefore, we here use LPS to stimulate amphioxus for detecting if *AmphiTAK1* is involved in innate immune response. We find that the expression level of *AmphiTAK1* is significantly increased at 10 h after LPS stimulation (Fig. 5). This expression pattern is also similar to other immune-related genes in amphioxus [35]. These results indicate that *AmphiTAK1* involves in innate immune response of *Branchiostoma belcheri*. In addition, to clarify that the expression of *AmphiTAK1* is associated with inflammatory response, we also simultaneously analyze the expression pattern of proinflammatory cytokine *AmphiTNF1* after LPS stimulation. We find that the expression level of *AmphiTNF1* is rapidly increased at 12 h after LPS stimulation. The expression levels of *AmphiTAK1* and *AmphiTNF1* are increased at 10 h and 12 h after LPS stimulation, respectively, indicating that *AmphiTAK1* is to some extent associated with inflammatory response.

Previous study has shown that TAB1 protein can interact with TAK1 protein in mammals [17]. The TAK1-TAB1 complex can lead to activation of downstream signaling factors including chemokines, proinflammatory cytokines and other immune factors [10,12,17,36–38]. Our previous work has obtained the full length cDNA sequence of *AmphiTAB1* from *Branchiostoma belcheri* (ID: KP938950), and found that the functional domain of AmphiTAB1 is also conserved by comparing with other species TAB1 proteins [39]. Specially, the expression level of *AmphiTAB1* is significantly increased at 6 h after LPS stimulation [39]. Therefore, our studies indicate that LPS stimulation could induce both TAK1 and TAB1 expression to involve in innate immune response of

Branchiostoma belcheri. Intriguingly, our present results have further demonstrated that AmphiTAK1 may interact with AmphiTAB1 by co-immunoprecipitation (Fig. 6). Taken together, our present works have revealed that AmphiTAK1 may interact with AmphiTAB1 to involve in innate immune response of *Branchiostoma belcheri*.

In summary, our present studies have identified *AmphiTAK1* as a member of the *TAK1* gene family, and revealed that AmphiTAK1 may interact with AmphiTAB1 to participate in innate immune response of *Branchiostoma belcheri*. Our results provide a new insight into innate immune function and evolution of *AmphiTAK1*.

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Appendix A. Supplementary data

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