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Identification, expression and antibacterial activity of a tachylectin-related homolog in amphioxus *Branchiostoma belcheri* with implications for involvement of the digestive system in acute phase response

Lingyan Ju, Shicui Zhang*, Yujun Liang, Xuemei Sun

Department of Marine Biology, Ocean University of China, Qingdao 266003, China

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ABSTRACT

Tachylectin-related proteins have been identified in various organisms from slime molds to sponges to bony fish, yet little is known to date about it in protochordate amphioxus, an important organism occupying a nodal position from invertebrates to vertebrates. Moreover, if the protein acts as an immune-relevant molecule remains controversial. Here we demonstrated the presence of a tachylectin-related gene in *Branchiostoma belcheri*. The predicted gene product, termed BbTL, consists of 305 amino acids with a putative N-terminal signal peptide and 6 tachylectin-typical tandem repeats of 30–33 amino acids. *In situ* hybridization histochemistry indicates a tissue-specific expression pattern of *BbTL* in adult amphioxus with the most abundant expression in the hepatic caecum and hind-gut. Quantitative real-time PCR reveals that challenge with LPS results in a significant up-regulation of *BbTL* expression in the guts. In addition, the recombinant BbTL expressed in *Pichia pastoris* is able to inhibit the growth of Gram-negative bacterium *Escherichia coli* in a dose-dependent manner. All these suggest that BbTL, like most other tachylectin-related proteins, is involved in the host immune defense, and the digestive system of *B. belcheri* appears the major immune tissue responding to LPS challenge.

1. Introduction

Lectins are carbohydrate-binding proteins or glycoproteins, which recognize specific sugar moieties and thereby agglutinate cells by binding to cell surface glycoproteins and glycoconjugates [1]. They are found in all types of living organisms, either in soluble or in membrane-bound form. Animal lectins serve many different biological functions including, in particular, the roles in the innate immune system by recognizing carbohydrates that are found exclusively on the surface of potential pathogens [2]. Similarly, the novel group of lectins identified recently, tachylectin-related proteins, has also been demonstrated to function in innate immunity from sponge to bony fish [3–7], but the hydroid tachylectin-related protein appears an exception [8].

Tachylectin-related proteins were first identified in horseshoe crab [3], *Tachypleus* (to which the name tachylectin refers), later in slime molds [9], and then in sponge [6], hydroid [8], carp [5], salmon [7] and zebrafish (GenBank accession number: AAM21310). They are structurally characterized by possessing six tandem repeats of 31–37 amino acids in length, forming a β-propeller structure [10]. Moreover, tachylectin-related proteins are related structurally to fibrinogen-like protein in that they display significant sequence similarity to the C-terminal globular domains of fibrinogen-like proteins, with approximately 35–50% identity [11]. Tachylectin-related proteins have been found to be present in the neurons of hydroids, the hemocytes and plasma of horseshoe crab, and the liver, head kidney, spleen and eggs of fish. However, information regarding the expression and function of tachylectin-related proteins in chordates remains very much limited.

Amphioxus or lancelet, a cephalochordate, has recently been regarded as the basal chordate [12], and is becoming an emerging model organism for insights into the origin and evolution of vertebrates [13]. In the course of expressed sequence tag (EST) generation from the gut cDNA library of adult amphioxus *Branchiostoma belcheri*, we isolated a gene fragment exhibiting identity to tachylectin-related genes. The purposes of this study were thus to examine the expression pattern of the tachylectin-related gene in adult amphioxus, to explore its expression regulation in response to challenge with lipopolysaccharide (LPS) and to test its antibacterial activity.

2. Materials and methods

2.1. Cloning and sequencing of BbTL cDNA

The gut cDNA library of amphioxus *B. belcheri* was constructed with SMART cDNA Library Construction Kit (Clontech, Palo Alto,

* Corresponding author. Tel.: +86 532 82032787.
E-mail address: sczhang@ouc.edu.cn (S. Zhang).

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CA, USA) as described previously [14]. In a large-scale sequencing of an amphioxus gut cDNA library with ABI PRISM 377XL DNA sequencer, more than 5000 clones were analyzed, which revealed one clone (No. E1559; length: 929 bp) displaying significant sequence similarity to tachylectins of other organisms. This EST sequence with 3′-end was therefore selected for further cloning of the full-length cDNA of *B. belcheri* tachylectin-related gene, *BbTL*.

Total RNAs were extracted with Trizol (Invitrogen) from adult *B. belcheri*, and polyA$^+$ RNA was purified using polyA tract mRNA isolation system II (Promega) according to the manufacturer's instructions. cDNA was synthesized from 5 μg polyA$^+$ RNA and linked with cassette adaptors by using M-MLV RTase cDNA Synthesis Kit (Takara) in combination with cDNA PCR Library Kit (Takara), and used as template. Two specific primers, C1 (reverse: 5′-AGACCTGGTTAGCGGTGGAGC-3′) and C2 (reverse: 5′-TAGTGG ATGTTACTCCTTGTC-3′), were designed based on the EST sequence obtained. The primary and nested PCR procedures were conducted by using C1, C2 and adaptor primer CA (forward: 5′-CGTGGTACCA TGGTCTAGAGT-3′) to amplify the 5′-end of BbTL cDNA. The primary PCR mixture (final volume 20 μl) contained 0.5 μl of cDNA mixture as template, 1× PCR buffer, 0.5 U of EX Taq DNA polymerase (Takara), 0.2 mM of dNTPs, 0.4 μM of the specific primer C1 and adaptor primer CA. The primary PCR amplification was carried out as follows: denaturation at 94 °C for 4 min, 32 cycles of 94 °C for 45 s/58 °C for 45 s/72 °C for 90 s and final extension at 72 °C for 7 min. The nested PCR amplification was performed in 20 μl of the reaction mixture containing 1 μl of 1:50 dilution of the primary PCR product as template, 1× PCR buffer, 0.5 U of EX Taq DNA polymerase, 0.2 mM of dNTPs, 0.4 μM of the specific primer C2 and adaptor primer CA. The PCRs were denaturation at 94 °C for 4 min, 32 cycles of 94 °C for 30 s/56 °C for 30 s/72 °C for 1 min, and final extension at 72 °C for 7 min. The nested PCR products of predicted sizes were purified using AXYGEM™ DNA Gel Extraction Kit (Axygen), ligated to the T/A cloning vector pGEM-T easy (Promega) at 4 °C overnight, and transformed into Top10 competent cells (Tiangen). Positive clones were selected, sequenced with ABI PRISM 3730 DNA sequencer, and the resulting sequences were subjected to the following analyses.

2.2. Sequence and phylogenetic analysis

BbTL cDNA sequence was analyzed using the BLAST algorithm at NCBI web site. SMART program (http://smart.embl-heidelberg.de/) was used to predict the functional sites or domains in the deduced amino acid sequence. The signal peptide was predicted with the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The potential sites of Asn-linked glycosylation were predicted with the NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc). The predicted protein sequence of *BbTL* was aligned with the sequences of 8 tachylectin-related proteins and analyzed for similarity with these known sequences using the MegAlign program (DNAStar) by the CLUSTAL W method.

A phylogenetic tree was constructed using the neighbor-joining method in the PHYLIP 3.67 software package (Department of Genome Sciences, University of Washington, Seattle). The tree was rooted with ORF_YEAST (*Pichia stipitis*, XP_001385352) as an outgroup and the tree reliability was evaluated by bootstrapping with 1000 replicates.

2.3. Northern blotting analysis

Total RNAs were prepared with Trizol (Invitrogen) from adult amphioxus ground in liquid nitrogen. An aliquot of 5 μg RNAs was electrophoresed and blotted onto Nylon membrane (Roche). The digoxigenin (Dig)-labeled *BbTL* riboprobes of about 750 bp were synthesized *in vitro* from linearized plasmid DNA following the DIG-UTP supplier's instructions (Roche). The membrane was prehybridized in DIG Easy Hyb buffer (Roche) at 55 °C for 3 h, and then hybridized at high stringency with 1 μg/ml *BbTL* riboprobes at 55 °C for 16 h, and washed twice in 2× SSC with 0.1% SDS at 25 °C for 5 min each and twice in 0.1× SSC with 0.1% SDS at 65 °C for 20 min each. They were subsequently incubated in a blocking solution containing 100 mM maleic acid (pH 7.5), 150 mM NaCl and 1% blocking reagent (Roche) and in the blocking solution with anti-Dig–alkaline phosphatase (AP) conjugated antibody (Roche) diluted 1:10,000 at room temperature for 1 and 2 h, respectively. After washing with 100 mM maleic acid buffer (pH 7.5) containing 150 mM NaCl and 0.3% Tween 20 and then with 100 mM Tris–HCl buffer (pH 9.5) containing 100 mM NaCl, the hybridized bands were visualized by BM-Purple (Roche).

2.4. In situ *hybridization histochemistry*

Sexually matured amphioxi were cut into three to four pieces and fixed in freshly prepared 4% paraformaldehyde in 100 mM phosphate-buffered saline (PBS; pH 7.4) at 4 °C for 8 h. The samples were dehydrated, embedded in paraffin, and sectioned at 6 μm. The sections were mounted onto poly-L-lysine-coated slides, dried at 42 °C for 36 h, and de-waxed in xylene for 20 min (two changes for 10 min each), followed by immersion in absolute ethanol for 10 min (two changes for 5 min each). They were re-hydrated, and finally equilibrated in double distilled H_2O containing 0.1% DEPC. *In situ* hybridization histochemistry was carried out as described by Fan et al. [15].

2.5. Quantitative real-time PCR

Real-time PCR was performed to investigate *BbTL* expression in response to challenge with LPS. Amphioxi *B. belcheri* collected from the "amphioxus ground" in the vicinity of Qingdao were acclimatized for three days, and then challenged with 10 μg/ml LPS (Sigma, MO, USA) in sterile seawater at room temperature, and sampled at 0 (control), 1, 3, 6, 12, and 24 h, respectively, after challenge. As *in situ* hybridization histochemical staining demonstrated that amphioxus tachylectin-related gene was mainly expressed in the hepatic caecum and hind-gut (see below), therefore the guts were dissected out of amphioxus *B. belcheri*. Total RNAs were prepared with Trizol from the guts and the gut-free body. After digestion with RQ1 RNase-free DNase (Promega) to eliminate the genomic contamination, cDNAs were synthesized with reverse transcription system (Promega) using oligo d(T) primer, and used as template. After qualification of the cDNA template, real-time PCR was performed on ABI 7500 real-time PCR system (Applied Biosystems, USA) to investigate the expression of *BbTL*. Two *BbTL*-specific primers, forward primer S1 5′-TCGTGTCTGTTGGGTCCA AG-3′ and reverse primer C2 5′-TAGTGGATGTTACTCCTTGTC-3′, were used to amplify a PCR product of 207 bp. β-Actin gene was chosen as the reference for internal standardization. Two *β-actin* primers, AF 5′-GAGACCTTCAACAGCCCAGC -3′ and AR 5′-CTCCAGAGTCCAGA CGATACC-3′, were used to amplify a β-actin gene fragment of 97 bp as the internal control for real-time PCR. The real-time PCR amplifications were carried out in triplicate in a total volume of 20 μl reaction mixture containing 10 μl 2× SYBR® *Premix Ex Taq*™ (Takara), 0.4 μl ROX Reference Dye II (50×), 1 μl of the 1:5 diluted cDNA, 0.2 μl each of S1 and C2 primers (20 μM) (or AF and AR to amplify *β-actin*) and 8.2 μl PCR-grade water. The real-time PCR program was denaturation at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s/60 °C for 15 s/72 °C for 35 s. Dissociation analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected.

After the PCR program, data were analyzed with 7500 System SDS Software v 1.4.0 (Applied Biosystems, USA). All analyses were