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Short communication

The molecular cloning and characteristics of a fibrinogen-related protein (TfFREP1) gene from roughskin sculpin (*Trachidermus fasciatus*)

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ABSTRACT

Fibrinogen-related proteins are a family of glycoproteins containing fibrinogen-like domains. Many members of these proteins play important roles in innate immune responses. We isolated a fibrinogen-related protein gene (*TfFREP1*) from roughskin sculpin (*Trachidermus fasciatus*). The *TfFREP1* encoded a protein of 264 amino acids, including 231 amino acids with fibrinogen-like domains. Both quantitative real-time polymerase chain reaction and western blot analysis showed that TfFREP1 was mainly expressed in skin and gill tissues of *T. fasciatus*. The expression level of TfFREP1 was upregulated at both mRNA and protein levels after stimulation of lipopolysaccharide. These results suggest that TfFREP1 may be involved in *T. fasciatus* immune reaction.

1. Introduction

Like other vertebrates, teleost fish species have an innate and adaptive immune system. However, the innate immune system is of prime importance for fish. Innate immunity refers to germline-encoded systems that discriminate non-self cells and various pathogens [1]. Lectins occupy an important position in this system. Lectins act against pathogens by aggregating and opsonizing them in vertebrate and invertebrate species, including fish. Some lectins contain fibrinogen-like (FBG) domains that are well known from vertebrate and invertebrate immune systems [2–4].

FBG domains consist of roughly 200 amino acid residues and exist in an increasing number of proteins [5]. Fibrinogen-related proteins (FREPs) are a family of glycoproteins that contain FBG domains. FREPs are highly conserved in this domain [6–8] and are universally found in vertebrates and invertebrates [9,10]. Many FREPs play important roles in innate immune responses. For example, the FBG domain of a tachylectins, a type of FREP and a unique lectin group from horseshoe crab (*Tachypleus tridentatus*), can bind and agglutinate all types of human erythrocytes and bacteria [10]. Recent findings in a range of invertebrate species have suggested that large clusters of FREPs play important and perhaps diverse roles in immune response [11–13]. Three FBG-containing proteins have been reported in teleost fish: fibrinogen (FB), angiopoietin (ANGPT), and microfibrillar-associated protein 4 (MFAP4) [14–18]. These proteins all contain a common C-terminal FBG domain. MFAP4 and FB beta chain genes may be involved in the innate immune response of fish [14,15]. However, to date, no relationship has been found between angiopoietin and fish immunity. In the present study, we discovered a fibrinogen-related protein (TfFREP1) gene from *Trachidermus fasciatus* (*T. fasciatus*). The TfFREP1 gene showed significant changes after injection with lipopolysaccharide (LPS) in skin and gill tissues. The newly detected protein gene may play a role in the teleost innate immune response.

2. Materials and methods

2.1. Immune challenge in fish

T. fasciatus were obtained from a fishery at Wendeng Bukou in Shandong Province, China and cultured in tanks filled with air-pumped seawater at 12 °C–14 °C for further analysis. The fish (30 g mean weight), were acclimated for at least one week before the immune challenge experiment. Subsequently, each fish was injected intraperitoneally with 0.1 mL of 0.9% NaCl solution (control group) or *Escherichia coli* LPS (Sigma) (experimental group) at 0.04 mg/kg. At 2, 6, 12 and 24 h post-injection, fish tissues from three control and three experimental fish were collected for further use.

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2.2. Total RNA isolation and cDNA synthesis

Total RNA was isolated from the above fish samples, including blood, gill, heart, kidney, intestine, liver, skin, spleen, stomach, and muscle, using an EZNA™ MicroElute® total RNA Kit II (QMEGA, USA) according to the manufacturer's instruction. The first-strand cDNAs were reverse transcribed following the SMART cDNA (BD Biosciences Clontech) method using the oligo-anchor R and Smart F primer, which are listed in Table 1.

2.3. Cloning of the full length cDNA of TfFREP

Based on the EST sequence of TfFREP1 obtained in our laboratory, specific primers (Tf1F1 and Tf1R1) were designed to clone TfFREP1. Subsequently, rapid amplification of cDNA ends was performed to obtain the full length of TfFREP1 sequence using 5′ primer/Tf1R1 and 3′ anchor R/Tf1F1. Polymerase chain reaction (PCR) was performed as follows: 94 °C for 3 min; 35 cycles at 94 °C for 35 s, 58 °C for 45 s, and 72 °C for 45 s; and 72 °C for 10 min. The PCR fragments were cloned into vectors and sequenced.

2.4. Sequence analysis

The similarity between TfFREP1 and other FREP-related proteins was analyzed using the online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast). The Expert Protein Analysis System (http://www.expasy.org) was used to analyze the amino acid sequence of TfFREP1. The FBG domain was predicted by SMART service (http://smart.emblheidelberg.de). Signal peptide was predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP). The multiple sequence alignment of amino acid sequences of corresponding FBG domains of TfFREP1 and other FREP-related proteins was created using the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw). A phylogenetic tree of selected FREP-related protein was constructed using the MEGA 4 program [19].

2.5. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) assay was performed using gene-specific primers (QTf1F and QTF1R) to study the tissue distribution and mRNA expression profile of the *TfFREP1* gene in skin and gill tissues at different times after the immune challenge with LPS (PCR product 180 bp). β-Actin was used as the internal control with the primers Actin F and Actin R. qRT-PCR was performed using SYBR Premix Ex Taq (Takara, Japan) following the manufacturer's instruction with a 7300 real-time system (Applied Biosystems, USA) and programmed at 94 °C for 3 min, followed by 40 cycles at 94 °C for 15 s, 60 °C for 60 s, followed by a final dissociation stage. All samples were repeated in triplicate. The relative expression levels of TfFREP1 response to LPS challenges were analyzed using the comparative CT method. The fold changes were calculated through $2^{-\Delta\Delta Ct}$ and statistically analyzed using an unpaired sample *t*-test. Significant difference was accepted at $P < 0.05$.

Table 1
PCR primers used in this study.

Primer name	Primer sequence (5′–3′)
Smart F	TACGGCTGCGAGAAGACGACAGAAGGG
Oligo anchor R	GACCACGCGTATCGATGTCGACT16(A/C/G)
5′ Primer	TACGGCTGCGAGAAGACGACAGAA
3′ Anchor R	GACCACGCGTATCGATGTCGAC
Tf1F1	TCTCAAGGCACAGACTGCACAC
Tf1R1	TTACACAGACTTGATCATCATTC
Actin F	TGAGACCACCTACAACAGCATC
Actin R	GAGCCTCCGATCCAGACAG
QTf1F	TGAGGGTTGACCTGTGGGAC
QTf1R	TGGTGCTGAAGCCGAAGC
Tf1BF	TACTCAGAATTCCAGAATGAGAAACCCCAGTG
Tf1BR	TACTCACTCGAGTTACACAGACTTGATCATCATTC

2.6. Recombinant expression and purification of TfFREP1 and antiserum preparation

Based on the cDNA fragment of TfFREP1, a pair of specific primers (Tf1BF and Tf1BR) was designed to amplify the sequence encoding the mature peptide of TfFREP1. An *Eco*R I site was added to the 5′ end of primer Tf1BF and an *Xho*I site to the 5′ end of primer Tf1BR after the stop codon. The amplified fragment was digested by the restriction enzymes *Eco*R I and *Xho* I, and then inserted into the expression vector pET-30a (+). The recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells for recombinant protein expression. The target protein was induced using 0.5 mM IPTG. The recombinant protein was purified using High-affinity Ni-IDA Resin (Gen-Script) according to the manufacturer instructions. The purified recombinant TfFREP1 was used to immunize rabbits as described previously [20] and blood samples were collected.

2.7. Western blot analysis of expression profiles of the TfFREP1 protein

Total proteins from the blood, gill, heart, kidney, intestine, liver, skin, spleen, stomach, and muscle were homogenized in a buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 3 mM EDTA, and 1 Mm PMSF). The samples were centrifuged at 10,000 rpm for 10 min at 4 °C to collect the supernatant. The concentration of total proteins was identified using the Bradford method, and bovine serum albumin (BSA) was used as standard [21]. Equal amounts of protein from the samples were loaded and separated via 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins on the gel were transferred to a polyvinylidene fluoride (PVDF) membrane blocked in 5% non-fat milk in TBS (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) for 2 h at room temperature. Subsequently, the membrane was incubated overnight with the 1/100 diluted antiserum to TfFREP1 in TBS. The antigen–antibody complexes were then visualized through a colorimetric reaction catalyzed by peroxidase-conjugated goat anti-rabbit IgG (1/10,000 diluted in TBS).

3. Results and discussions

3.1. Cloning and sequence analysis of TfFREP1

FREPs function as lectins involved in the innate immune defense of invertebrates and vertebrates [8,10,22,23]. In this study, we first attempted to characterize TfFREP1, a kind of FREP, in scorpaeniformes fish. The full length of TfFREP1 cDNA (accession number JQ670875) was 1047 bp with an open reading frame (ORF) of 795 bp. The ORF of TfFREP1 encoded a putative protein of 264 amino acids consisting of a signal peptide (1–24 amino acids) and a conserved FBG domain (34–264 amino acids). This putative protein had one potential Asn-linked glycosylation site at residual 103 and two potential calcium-binding sites in the latter half of the C-terminal (Supplementary Fig. 1). The mature TfFREP1 protein had a theoretical molecular weight of approximately 26.7 kDa and an isoelectric point of 5.71. BLASTp searching at NCBI revealed that TfFREP1 had an FBG domain, and its N-terminal region did not match any known protein sequence. Multiple sequence analysis revealed that the FBG domain of TfFREP1 shared 30%–44% identity