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Identification and characterization of a novel PRR of fibrinogen-related protein in *Apostichopus japonicus*

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ABSTRACT

Fibrinogen-related proteins (FREPs) play important roles in innate immunity by recognizing pathogen associated molecular patterns on pathogenic bacteria surfaces via conserved fibrinogen-like domain (FBG). In this paper, the full-length cDNA of *Apostichopus japonicus* FREP (designated as *AjFREP*) was cloned combined with rapid amplification of cDNA ends (RACE) and transcriptome sequencing. The full-length cDNA of *AjFREP* was of 2110 bp with an open reading frame (ORF) of 1659 bp. SMART analysis revealed that the AjFREP contained a typical signal peptide of 19 amino acid residues, a FBG and two unusual epidermal growth factor-like domains (EGFs). Multiple sequence alignments suggested that FBG domain shared a remarkably high structural conservation in polypeptide binding site and Ca^{2+} binding site. Tissue distribution analysis revealed that *AjFREP* was constitutively expressed in all examined tissues with the largest magnitude in coelomocytes, indicating *AjFREP* might play an important role in immune defense. The mRNA level of *AjFREP* in coelomocytes was sharply up-regulated by *Vibrio splendidus* challenge, and reached its peak expression at 48 h. Knock-down *AjFREP* by specific siRNA could significantly repress the coelomocyte phagocytosis rate. Meantime, the survival number of *V. splendidus* in the coelomic fluid was promoted. All these current results indicated that *AjFREP* might be involved in pathogen clearance through mediating coelomocytes phagocytosis activity.

1. Introduction

The innate immune system is activated by the so-called pattern recognition receptors (PRRs), which can recognize the distinct pathogen associated molecular patterns (PAMPs) [1,2]. Nowadays, more than 10 kinds of PRRs are found in invertebrates, including FREPs, peptidoglycan recognition proteins (PGRPs) [3], Gram-negative binding proteins (GNBP) [4], lipopolysaccharide and β-1,3-glucan binding proteins (LGBPs) [5,6], C-type lectins [7], galectins [8], thioester-containing proteins (TEPs), scavenger receptors (SRs), down syndrome cell adhesion molecules (DSCAMs) and Toll like receptors (TLRs), and their connections with an innate immune responses are also intensively investigated in vertebrates [9]. Increasing evidences indicate that FREP (also known as FBN) has emerged as being important components of the innate immunological response of invertebrates with pathogen recognition and complement activation [10–12].

FREPs universally exist in both invertebrates and vertebrates, which includes the eponymous fibrinogen as well as the tenascin, angiopoietin, and ficolin families [13]. Among them, ficolins receive more attention [14]. Ficolins are a group of proteins which consist of a collagen-like domain and a FBG. In humans, ficolins primarily divided into M-ficolin (also called Ficolin-1 or Ficolin/P35-related protein), L-ficolin (also called liver ficolin, Ficolin-2 or Ficolin/P35), and H-ficolin subgroups (also called Ficolin-3 or Hakata antigen) [15]. M-ficolin has highest specificity for 9-O-acetylated sialic acid in a α2-6 linkage to galactose [16]. L-ficolin is a unique plasma recognition molecule with a broad specificity for microorganisms [17]. H-ficolin revealed the highest capacity in complement activating compared with M-ficolin and L-ficolin, and was highly resistant to bacterial collagenase treatment [18]. Compared with ficolins, FBG-containing proteins lack collagen-like domain in N terminus [19]. In invertebrates, many FREPs were also identified from different species such as mollusc (*Biomphalaria glabrata*; *Argopecten irradians*), echinodermata (*Strongylocentrotus purpuratus*), arthropods (*Pacifastacus leniusculus*; *Marsupenaeus japonicus*), and brachiopods (*Lingula anatina*) [20,21]. They commonly contain a FBG domain in the C terminal [22]. The FBG domain with roughly 200 amino acid residues [23] is highly conserved in all FBG domain-containing proteins, which is responsible for carbohydrate-binding and pathogen-binding with the conserved hydrophobic residues [24,25]. Their functions in invertebrates have been shown in immune defense,

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such as pathogen recognition and bacterial defense [26].

The invertebrate sea cucumber (*Apostichopus japonicus*) (Echinodermata, Holothuroidea) with an innate immune system is a commercially important species in Chinese marine culture [27]. Unfortunately, the intensification and rapid expansion of *A. japonicus* farming has led to outbreaks of infectious diseases [28]. Skin ulceration syndrome (SUS) was one of the most contagious and lethal disease in sea cucumber culture industry [29]. To establish a highly effective disease control strategy, identification of novel immune-related genes in this specie seems urgent, particularly the fibrinogen-related protein, which plays a critical role in host defense against bacterial infections [30]. To fill the gap, we cloned a new fibrinogen-related protein and characterized its expression profiles towards *V. splendidus* challenge in this study. Phagocytosis activities and bacterial clearance capacities were also investigated in coelomocytes and coelomic fluid separately after *AjFREP* silencing. Our present work would provide basic information to understand the versatile roles of FREP in immune response.

2. Materials and methods

2.1. Experimental animals and challenge studies

Healthy adult sea cucumbers *A. japonicus* (weight 115 ± 13 g) were obtained from Dalian Pacific Aquaculture Company (Dalian, China) and acclimatized in 30 L aerated natural seawater (salinity 28, temperature 16 ± 1 °C) for 3 days. For the immune challenged experiment, sea cucumbers were randomly divided into six tanks with each containing 10 individuals. One tank was served as control group, and the other five tanks were immersed with high density of *V. splendidus* at the final concentration of 10^7 CFU mL^{-1}. After challenge, coelomic fluids were collected at 0, 6, 24, 48, 72, and 96 h and centrifuged at 800 × g for 5 min at 4°C to harvest coelomocytes. Five biological replicates were obtained for each sampling time-point. For spatial expression analysis, coelomocytes and other four tissues including muscle, tentacle, respiratory trees and intestine were collected from healthy individuals using sterilized scissors and tweezers. These tissues were ground into powder in liquid nitrogen using a sterile mortar and a pestle. We performed five replicates for each tissue. The samples were stored at −80 °C for RNA extraction and cDNA synthesis.

2.2. Cloning and sequence analysis of AjFREP

Total RNA was extracted from the tissues of *A. japonicus* with RNAiso plus reagent (TaKaRa), and treated with RNase-free DNase I (TaKaRa) to remove the genomic DNA. Then the first-strand cDNA was synthesized according to the Primescript™ II 1st cDNA Synthesis Kit (TaKaRa) following the manufacture's protocol. The gene specific primers of *AjFREP* (Table 1) were designed based on the corresponding unigenes in the transcriptome data [31]. The full-length cDNA sequence of *AjFREP* was subsequently assembled by using the 3′, 5′-Full RACE Kit (TaKaRa) following the manufacturer's instructions. The desired PCR products were purified and then cloned into the pMD19-T simple vector (TaKaRa). The ligation product was transformed into *Escherichia coli* DH5α (TaKaRa) and three positive clones for each product were sequenced at Sangon (Shanghai, China). The *AjFREP* cDNA sequence was analyzed using the BLAST algorithm at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence of AjFREP was analyzed with the expert protein analysis system (http://www.expasy.org/). The AjFREP domain features and signal peptide was predicted with the Simple Modular Architecture Research Tool (SMART) program (http://www.smart.emblheidelberg.de/) and multiple sequence alignments were created using DNAMAN software. A neighbor joining (NJ) tree was constructed by Mega 5.0 software package (http://www.megasoftware.net/) and Clustal X (1.83) with 1000 bootstraps.

Table 1
PCR primer sequences and siRNA information in this study.

Primer Name	Target	Primer Sequence (5′-3′)
AjFREP 3-1	3′ RACE	TACCTCCGACGGGACACCTA
AjFREP 3-2		AAGCGGTTGGTGGCATTC
AjFREP 5-1	5′ RACE	GGAATCCAAATCCACCTTCAT
AjFREP 5-2		ATGACCGTCCATCCTCCTG
AjFREP qF	Real-time PCR	TACCTCCGACGGGACACCTA
AjFREP qR		GGAAGTTACCGCAAGCATCA
AjFREP F	FBG amplification	GAACCTTATGAAGACTGTAAAGA
AjFREP R		TCATGAAGAAGGTCGAATTTTCA
AjFREP EcoRI F	Vector construction	GAATTCGAACCTTATGAAGACTGTAAAGA
AjFREP XhoI R		CTCGAGTCATGAAGAAGGTCGAATTTTCA
AjFREP sense	RNAi	CCAGUGGAGUAUACCUCAUTT
AjFREP anti-sense		AUGAGGUAUACUCCACUGGTT
*Ajβ-actin*F	Real-time PCR	CCATTCAACCCTAAAGCCAACA
*Ajβ-actin*R		ACACACCGTCTCCTGAGTCCAT
NC sense	RNAi	UUCUCCGAACGUGUCACGUTT
NC anti-sense		ACGUGACACGUUCGGAGAATT

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1                                                    M  E  F  L  L  L  V  S  A
1   GAAAATTCCTGATCTTCTTCGAAAGTCAAAAGGATGGAGTTTCTCTTGCTTGTAAGCGCA
10  V  F  A  F  S  C  T  T  I  A  N  V  P  S  S  D  Q  T  S  N
61  GTTTTTGCATTTTCGTGTACAACGATTGCCAACGTTCCGTCGTCAGATCAAACGAGTAAT
30   H  Q  H  S  L  P  S  T  T  D  D  T  Q  S  G  S  Y  F  F  Y
121 CATCAGCACAGCCTGCCTTCAACTACAGATGACACTCAAAGTGGATCGTATTTCTTTTAT
50   Q  S  S  E  Y  P  K  D  C  R  D  V  Q  R  Q  C  S  T  S  S
181 CAAAGTTCTGAATATCCCAAGGACTGTCGAGATGTCCAACGTCAGTGTTCTACATCAAGT
70   S  S  G  V  Y  L  I  K  P  D  G  Y  K  E  P  F  E  V  F  C
241 TCCAGTGGAGTATACCTCATCAAACCAGATGGTTATAAAGAACCATTTGAAGTATTCTGC
90   N  N  D  V  D  T  G  G  W  T  V  I  Q  R  R  E  G  G  F  V
301 AACAACGATGTAGACACAGGAGGATGGACGGTCATTCAACGACGTGAGGGGGCTTTGTG
110  N  F  N  R  S  W  A  Q  Y  E  G  G  F  G  F  L  S  S  E  F
361 AATTTTAACAGGAGTTGGGCACAGTATGAAGGTGGATTTGGATTCCTGTCCTCTGAGTTT
130  W  L  G  N  E  K  L  S  Y  L  T  N  Q  E  V  Y  E  L  R  V
421 TGGCTCGGCAATGAAAAGTTGTCGTATTTGACGAATCAGGAAGTGTATGAACTTCGTGTG
150  D  I  T  L  Y  N  G  S  A  L  Y  A  I  Y  K  G  F  R  I  T
481 GATATTACTCTTTATAATGGGTCAGCCTTGTATGCTATTTACAAAGGTTTCCGTATCACT
170  D  G  W  S  Q  Y  M  I  S  S  I  G  V  L  E  S  N  L  G  S
541 GACGGATGGAGTCAATATATGATATCCAGTATAGGAGTATTGGAAAGCAATTTAGGCTCA
190  A  L  S  I  C  P  S  N  M  I  Y  N  T  C  S  C  Q  A  T  C
601 GCACTGTCAATCTGTCCTTCGAATATGATCTACAACACCTGCAGTTGCCAAGCAACGTGC
210  D  D  P  N  G  A  G  G  C  N  N  N  C  L  G  S  E  G  C  N
661 GATGATCCCAATGGAGCGGGTGGATGTAATAATAACTGCCTGGGAAGTGAGGGTTGTAAC
230  C  P  T  G  F  L  M  H  G  S  D  C  I  P  T  S  D  C  G  C
721 TGTCCTACAGGGTTCTTAATGCATGGAAGTGACTGCATACCTACGAGTGACTGTGGTTGT
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Fig. 1. The cDNAs and deduced amino acid sequences of *AjFREP*. The start codon was blacked. The asterisk indicated the stop codon. The signal peptide was indicated in box. The EGF was shown in gray shadow and FBG in the C-terminal was underlined. The RNA instability sequences (ATTTA) and polyadenylation signal (AATAAA) were bolded and italics.

2.3. Quantitative real-time PCR analysis of AjFREP mRNA expression

The tissue distribution and time-course expression of *AjFREP* were performed using an Applied Biosystem 7500 fast Real-time PCR System. For normalizing the *AjFREP* transcripts, *Ajβ-actin* was amplified as an