Fish & Shellfish Immunology 32 (2012) 307–315

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

A novel protein with a fibrinogen-like domain involved in the innate immune response of *Marsupenaeus japonicus*

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ARTICLE INFO

Article history:
Received 31 August 2011
Received in revised form
25 October 2011
Accepted 20 November 2011
Available online 27 November 2011

Keywords:
Fibrinogen-related protein
Marsupenaeus japonicus
Fibrinogen-like domain

ABSTRACT

Fibrinogen-related proteins play important roles in innate immunity. We isolated a fibrinogen-related protein gene (*MjFREP1*) in kuruma shrimp *Marsupenaeus japonicus*. *MjFREP1* encoded a protein of 270 amino acids, including a 223 amino acid fibrinogen-like domain. Quantitative real-time polymerase chain reaction analysis shows that *MjFREP1* is mainly expressed in the gills and the expression is significantly upregulated by *Vibrio anguillarum, Staphylococcus aureus*, or white spot syndrome virus (WSSV) challenge. Recombinant MjFREP1 fibrinogen-like domain agglutinates Gram-positive bacteria *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus megaterium*, and *S. aureus* in the presence of calcium ions. The fibrinogen-like domain of MjFREP1 binds peptidoglycans, LPS, bacteria, and the VP28 of WSSV. These results suggest that the MjFREP1 may play an important role in the shrimp immune response against different pathogens.

1. Introduction

Innate immunity critically relies on first-line host defense molecules to discriminate various pathogens. Shrimp immune response is governed mainly by mechanisms of innate immunity where pattern-recognition receptors (PRRs) play an important role in the first-line of immune defense [1]. Lectins are a set of the most common PRRs in innate immunity. Several lectins containing C-terminal fibrinogen-like (FBG) domains act as PRRs in invertebrates [2].

Fibrinogen-related proteins (FREPs) are a family of proteins containing FBG domains and are found universally in vertebrates and invertebrates [3,4]. In invertebrates, FREPs are a common PRR family involved in immune responses. The FBG domain consists of approximately 200 amino acid residues, among which 24 are invariant and mostly hydrophobic [5]. The FBG domain is highly conserved in all FBG domain-containing proteins and is responsible for carbohydrate-binding and pathogen-binding [6].

Several kinds of FREPs have been found in various invertebrate species and many members of this family are likely involved in innate immune response and act as PRRs. For example, tachylectins TL5A and TL5B from the horseshoe crab *Tachypleus tridentatus* can specifically bind to N-acetylglucosamine (GlcNAc) and agglutinate all types of human erythrocytes and bacteria [4]. FREPs identified from the slug *Limax flavus* binds to non-self antigens [7], and a family of FREPs from snail *Biomphalaria glabrata* recognizes different categories of pathogens and parasites [8]. BbFREP in amphioxus *Branchiostoma belcheri* is a multivalent PRR with strong bacteriolytic activity against both the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Staphylococcus aureus* [9]. FREP in bay scallop *Argopecten irradians* functions as a PRR in the immune response [10]. The FBG domains of the FREPs from the mosquito *Armigeres subalbatus* (also called aslectin) binds to GlcNAc and bacteria in the host immune response [11]. The FREPs in the mussel *Mytilus galloprovincialis* are stimuli-dependent based on pathogen challenges [12]. Moreover, a large number of fibrinogen-like loci were found in invertebrate genomes recently [13]. All these findings suggest that fibrinogen-like proteins play important and perhaps diverse functions in the invertebrate immune response.

A large number of the FREP family have been found in invertebrates and shown to be important components of the invertebrate immune system. In crustaceans, only four FBG-containing proteins have been reported recently: two melanization-inhibiting proteins from the crayfish *Pacifastacus leniusculus* and the shrimp *Penaeus monodon* and two novel ficolin-like proteins from *P. leniusculus* [14–16]. In the present study, a new FREP (MjFREP1) from *Marsupenaeus japonicus* was found. Our results suggest that this protein may be involved in immune responses against different pathogens.

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doi:10.1016/j.fsi.2011.11.020

2. Materials and methods

2.1. Chemicals and microorganisms

Unizol was obtained from Biostar (Shanghai, China). RevertAid First Strand cDNA Synthesis Kit was a product of Fermentas (Burlington, Canada). Peptidoglycan (*Micrococcus luteus*) was obtained from Sigma (St. Louis, MO, USA). *Bacillus subtilis*, *Bacillus megaterium*, *S. aureus*, and *Bacillus thuringiensis* were obtained from the Shandong Agricultural University. *Vibrio anguillarum* was a gift from the Institute of Oceanology, Chinese Academy of Sciences. *E. coli* and *Saccharomyces cerevisiae* were available in our laboratory.

2.2. Immune challenge in shrimps

Kuruma shrimp, *M. japonicus*, were obtained from a market in Jianan, Shandong Province, China and cultured in tanks filled with air-pumped seawater in the laboratory. In the immune challenge experiments, the abdominal segment of each shrimp was injected with 2×10^7 cells of *V. anguillarum, S. aureus* or 3.2×10^7 copies of white spot syndrome virus (WSSV) which were extracted from the gills of naturally heavily infected *Fenneropenaeus chinensis* that had been stored at −80 °C [17]. At 2, 6, 12 and 24 h post-injection, hemolymph was collected from the ventral sinus using 1/10 volume of anticoagulant (10% sodium citrate, pH 7.0) with 200 mM phenylthiourea as a melanization inhibitor [17]. The hemolymph was then centrifuged at 800×g for 5 min at 4 °C to isolate the hemocytes. The hearts, hepatopancreas, gills, stomachs, and intestines were also collected for RNA extraction at 0, 2, 6, 12, and 24 h post-injection.

2.3. Total RNA isolation and cDNA synthesis

Total RNA from hemocytes and other tissues (heart, hepatopancreas, gills, stomach, and intestine) was extracted using Unizol reagent (Biostar, China). The first-strand cDNAs were reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada).

2.4. Cloning of the full-length cDNA of MjFREP

Based on the expressed sequence tag (EST) sequence of *MjFREP1* obtained in our laboratory, specific primers were designed to clone the full-length of *MjFREP1*. The primers Mj1F1: GAGGAGGAGAATGAACTCAA and Mj1R1: TTGACCTCCTTCAGGCTG were used to clone the 5′-end, and the primers Mj1F2: CGG GAG TAT TGA TGC GGA and Mj1R2: TTA AAC ACG CGG TTT TCA were used to clone the 3′-end of the gene. The polymerase chain reaction (PCR) was performed as follows: 94 °C for 3 min; 35 cycles (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.

2.5. Sequence analysis

The consensus sequence of MjFREP1 and other FREP-related proteins was analyzed using the online BLAST algorithm (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 MjFREP1. The FBG domain structure was predicted by the SMART program (http://smart.emblheidelberg.de/). The multiple sequence alignment of the amino acid sequences of the corresponding FBG domains of MjFREP1 and other FREP-related proteins were created using the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). The phylogenetic tree of selected FREP-related proteins was constructed using the MEGA 4 program [18].

2.6. Quantitative real-time PCR

A quantitative real-time PCR (qRT-PCR) assay with gene-specific primers (Mj1F3: GAG AGG GCT TCG GGA GTA TTG and Mj1R3: GTC GCC AAG GGT GCT GTT C) was performed to study the tissue distribution and mRNA expression profile of *MjFREP1* in the gills at different times after the immune challenge with *V. anguillarum*, *S. aureus* and WSSV (PCR product: 214 bp). β-Actin transcripts (MactF: AGT AGC CGC CCT GGT TGT AGA C, MactR: TTC TCC ATG TCG TCC CAG T) was used as the internal control. The qRT-PCR was performed using a real-time thermal cycler (Bio-Rad, USA) and programmed at 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s, 72 °C for 20 s, and melting from 60 to 95 °C. The mRNA expression level of the *MjFREP1* responding to *V. anguillarum, S. aureus,* and WSSV challenges were calculated using $2^{-\Delta\Delta Ct}$ and subjected to statistical analysis using an unpaired sample *t*-test. The significant difference was considered if $P < 0.05$.

2.7. Recombinant expression and purification of the FBG domain of MjFREP1 and antiserum preparation

The cDNA fragment encoding the FBG domain of MjFREP1 was amplified with specific primers (Mj1F3: TAC TCA GAA TTC ATG CGA CCA GCC AAC TGC, Mj1R3: TAC TCA CTC GAG TTA CTG AGG CCG GAA CAT GAA). An *Eco*RI site was added to the 5′-end of primer Mj1F3 and an *Xho*I site to the 5′-end of primer Mj1R3 after the stop codon. The DNA fragment was digested by restriction enzymes *Eco*RI and *Xho*I, then inserted into the expression vector pGEX 4T-1 [adding a 27 kDa glutathione S-transferase (GST) tag to target protein]. The recombinant plasmid was transformed into *E. coli* BL21 cells. The vector pGEX 4T-1 without any inserts was selected as a negative control. The target protein of positive transformants and negative control were induced by 0.5 mM IPTG and purified by Glutathione Sepharose 4B chromatography (Novagen, Merck Group). The purified FBG domain of MjFREP1 and negative control GST were used as antigens for antiserum preparation following the method described in the literature [19].

2.8. Bacterial agglutination assay

Agglutination assay was performed with Gram-positive bacteria (*B. megaterium*, *B. thuringiensis*, *B. subtilis*, and *S. aureus*) and Gram-negative bacteria (*V. anguillarum*, and *E. coli*). Mid-logarithmic phase bacteria were collected by centrifugation at 6000×g for 5 min and resuspension in Tris-buffered saline (TBS) to 2×10^8 cells/ml. Microorganisms were incubated with 25 μl of TBS containing different concentrations (12.5–200 μg/ml) of the FBG domain of MjFREP1 protein in the presence or absence of 10 mM $CaCl_2$. The mixture was incubated at room temperature for 1 h and observed for agglutinating reactions under a microscope. GST at 200 μg/ml containing 10 mM $CaCl_2$ was used as a negative control.

2.9. Bacterial binding assay

Bacterial binding analysis was performed as described previously [18]. Gram-positive bacteria (*B. megaterium*, *B. thuringiensis*, *B. subtilis*, and *S. aureus*), Gram-negative bacteria (*V. anguillarum* and *E. coli*), and yeast (*S. cerevisiae*) were used. A 1 ml sample of late-logarithmic phase cultures of bacteria were pelleted by centrifugation at 6000×g for 5 min, washed with TBS, and resuspended in TBS to OD600 of 1.0. The purified recombinant MjFREP1 was incubated with microorganisms with rotation for 20 min at