Fish & Shellfish Immunology 30 (2011) 434–438

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

A new gene family of single fibrinogen domain lectins in *Mytilus*

A.M. Gorbushin*, N.V. Iakovleva

Institute of Evolutionary Biochemistry and Physiology RAS, pr. Torez 44, Saint-Petersburg 194223, Russian Federation

ARTICLE INFO

Article history:
Received 5 May 2010
Received in revised form
17 September 2010
Accepted 7 October 2010
Available online 15 October 2010

Keywords:
Innate immunity
Lectins
Pattern-recognition receptors
Phylogeny
Mollusca
Bivalvia

ABSTRACT

In molluscs haemolymph lectins bearing fibrinogen-like domain (FREP) act as immune pattern-recognition receptors. A full-length cDNAs of MytFREP1 and MytFREP2 cloned from haemocytes of blue mussel *Mytilus edulis* encoded putative polypeptides of 230 and 241 amino acids. Both polypeptides consist of signal peptide and C-terminal fibrinogen-like domain. Immune functions of these molecules may be extrapolated from the close-related and functionally characterized lectin AiFREP from bay scallop, *Argopecten irradians*. However, immune challenge experiments with zymosan particles, *Escherichia coli* bacterium and cercariae of *Himasthla elongata* (Trematoda) failed to modulate MytFREP1 and MytFREP2 mRNA expression in *M. edulis* haemocytes. Hypothetically, it argues into rather high specificity of mechanisms triggering a differential expression of MytFREP genes. The search in the EST database revealed orthologous copies for described genes and portion of relatively similar genes from two close-related mytilids, *Mytilus galloprovincialis* and *Mytilus californianus*. We document the new multigene family of FREPs from bivalves of genus *Mytilus*. MytFREP family currently represented by 2 genes from *M. edulis*, 4 genes from *M. californianus* and 7 genes from *M. galloprovincialis*.

1. Introduction

The management of Protostomata immune responses is governed by mechanisms of innate immunity in that pathogen-associated molecular patterns are recognized by pattern-recognition receptors (PRR). Lectins as a group of carbohydrate-binding proteins is most common PRRs acting throughout specific attachment themselves to the more or less unique combination of carbohydrate moieties exposed on the invader cell surface [1]. Lectins containing C-terminal fibrinogen-like domain (FBG) are well-known from vertebrate immune systems [2], however interest to their roles in invertebrate immunity has increasingly developed in the last decade.

The molecular nature of FBG-related proteins (FREPs) in molluscs was discovered in 1997 [3] and current insight into FREP functions suggests that these lectins have the ability to recognize a wide range of pathogens including bacteria, yeast and multicellular parasites, such as trematodes. This, together with the dynamic changes of multiple IgSF-FREP proteins in plasma of *Biomphalaria glabrata* after exposure to trematode parasites and upregulation of AiFREP expression in *Argopecten irradians* after challenge with Gram-negative marine bacteria suggests that these molecules play a significant role in immune defense of molluscs [3–5]. Since discovering FREPs in *B. glabrata* considerable progress has been made in terms of manifesting gene structure, polymorphism, mRNA, protein expression and functions of these lectins. However, one of the shortcomings in our current knowledge of FREPs is the lack of relevant data sufficient for adequate comparative analysis necessary to provide insights into molecular and functional evolution of these proteins. To help to overcome this gap we introduce in the practice of PRR-oriented immunological investigations the well-known, wide-spread albeit still unexploited from this point of view the group of marine bivalves of genus *Mytilus*.

The mussels of this genus are represented by four species, *Mytilus californianus*, *Mytilus edulis*, *Mytilus galloprovincialis* and *Mytilus trossulus*. The purely Pacific species *M. californianus* is morphologically and genetically rather different from other members of the genus [6]. The commercially important *M. edulis, M. galloprovincialis* and *M. trossulus* are genetically more similar to each other and are hardly distinguishable by shell morphology. All three species are found in the North Atlantic, where their distribution ranges overlap and a hybrid zones are formed [7]. To date only *Mytilus edulis* (blue mussel) have been recorded in the White Sea [8].

The goals of this study were 1) to clone the full-length cDNAs of the FREP lectins from the White Sea *M. edulis*, 2) to examine mRNA expression pattern of these proteins in haemocytes after immune challenges, 3) to define the new group of orthologous FREP genes from *Mytilus* genus.

* Corresponding author.
E-mail address: agorbushin@gmail.com (A.M. Gorbushin).

1050-4648/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2010.10.002

2. Materials and methods

Standard molecular biology procedures were used for cDNA cloning and sequencing as described by [9]. Total RNA was isolated from the haemocytes of blue mussel *M. edulis* using TRIzol reagent (Invitrogen). For all RT-PCR applications the DNase I (Promega)-treated RNA was used as template to avoid false-positive results due to genomic DNA contamination. The cDNA of *M. edulis* FREPs was cloned by rapid amplification of cDNA ends (RACE [10]) method. For this, two sequence fragments were cloned and sequenced preliminary as a result of RT-PCR with degenerate primers (FREPs-1[CH] GTGGAAGGAGTACAGGSANGGNTTYGG and FREPas-1[CH]L GTCGTTGTCGTGGTCCTTNGTNSWRAA).

All blue mussels were of 8 cm shell-length, collected from cultured settlements near the White Sea Biological Station "Kartesh" (WSBS; Kandalaksha Bay of the White Sea). Two immune challenge experiments were carried out in order to estimate MytFREP1 and MytFREP2 mRNA expression in haemocytes sampled with haemolymph from posterior adductor sinus using syringe. The first experiment, *in vitro*, was performed in three replicates with haemolymph of 10 mussels in each pooled together and then aliquoted in fifteen 1 ml samples. Each of 5 samples in three batches, the control and two experimental, received respectively 100 μl of sterile seawater (SSW), *Escherichia coli* bacterium, zymosan particles in SSW (OD600 = 0.5, pH 7.8) and kept at 18 °C until the end of experiment. Haemolymph from each treatment was centrifuged at one of the five time points (1, 3, 6, 9 and 12 h) and harvested blood cells were subjected to RNA isolation and cDNA synthesis as described above. The second experiment, *in vivo*, was performed by injection into the cavity at the base of mussel foot with 300 μl of the zymosan particles in SSW and 40–50 alive cercariae of *Himasthla elongata* (Trematoda: Echinostomatidae) emitted throughout 2 h from the three infected common periwinkles, *Littorina littorea*. *H. elongata* exploits the blue mussel as a second intermediate host. Due to high toxicity of *Mytilus* haemolymph, cercariae should encyst in the host tissues within 2 h, otherwise the risk of the larvae injury by humoral and cellular components of the mussel haemolymph would increase dramatically [11]. Six individual mussels in each treatment and six control ones injected with SSW were placed in aerated seawater tanks for 6 h at 18 °C. Then, the haemolymph from these 18 mussels was sampled, centrifuged, and haemocytes were subjected to RNA isolation and cDNA synthesis.

Semiquantitative duplex-RT-PCR assay with gene-specific primers (MytFrep1s: TGCGAATGCAGAGCTTCCTAGGG, MytFrep1as: GTCCGCT GAATGCATCACCTGC, MytFrep2s: AGAGGGATTCGGGACTCTTCAGG, MytFrep2as: GCCTCCGTACCACCATCCGG) was applied to estimate mRNA expression of MytFREP1 and MytFREP2 genes (PCR products 419 and 322 bp, respectively) and β-actin transcripts (MactF: ATGACATGGAGAAGATCTGGC, MactR: CCAAGGAAAGATGGCTGGAACA; product 565 bp) as a control of total mRNA quality and loading. The resulting PCR products were visualized on a 1.8% agarose gel. Gel images were digitally captured and product optical density was determined using the Gel-Pro software (Media Cybernetics). Mean estimates of values, presented as a ratio of the MytFREP signal divided by the actin signal, from control and experimental treatments were compared using the two-tail *T*-test for independent measurements.

2.1. Sequence analysis

Using a combination of methods, the predicted protein sequences were screened to confirm the presence of fibrinogen C-terminal domain-like superfamily domain (FreD): (1) blast search against the NCBI protein database resulted in a significant hit to a FreD containing sequence and (2) protein domain search against SMART database [12]. Putative signal sequences were identified by SMART and confirmed by hydropathy plots. Molecular weight and theoretical pI were computed using ProtParam tool (ExPASy). The phylogenetic relationships of FREP nucleotide sequences were determined by using Neighbor-Joining method with *Gamma* parameter 1.0 in Mega4 [13]. Bootstrap support values after 5000 replicates were obtained for the NJ trees.

3. Results

3.1. Characterisation of M. edulis FREP cDNAs

A full-length cDNAs of *M. edulis* FREP1 and FREP2 (816 and 835 bp respectively) were composed of a 690 and 723 bp open reading frames that translate into a putative peptides of 230 and 241 amino acids. There are a 30 and 43 bp long 5′ untranslated regions (5′UTR) and 69 and 37 bp 3′ UTR. The nucleotide sequences of *M. edulis* FREP1 and FREP2 are 53% identical and according to cut-off point of <86% are the different genes (subfamilies). The sequences were assigned as MytFREP1 and MytFREP2 (GenBank accession numbers HM147143 and HM147144).

Basic physicochemical and comparative characteristics of predicted *M. edulis* MytFREP1 and MytFREP2 proteins are given in the Table 1. Both putative polypeptides consist of signal peptide and C-terminal FBG domain (Fig. 1) which is highly conserved. SMART library searches classify those in both proteins as FBG domains with high scores (E-value $< 10^{-80}$). On Fig. 1 the alignment of the *M. edulis* FREPs and AiFREP from bay scallop, *A. irradians* is shown. Of 24 canonical residues observed in other FBG domains, the FBG domains of both MytFREPs have all present. From a structural modelling perspective, the *MytFREPs* has predicted homology to human M-ficolin fibrinogen-like domain (PDB: 2D39; $E = 6.0^{-51}$) with known crystal structure.

MytFREP1 and MytFREP2 were constitutively expressed in blue mussel haemocytes at relatively high level. In the *in vitro* experiment, expression decreased with a time only in the zymosan treatment (Fig. 2 A). In the *E. coli* and the control treatments, transcription pattern was uniform throughout the experimentation suggesting that physiological state of pooled haemocytes *in vitro* was appropriate even in 12 h after sampling. However, no significant ($P > 0.05$) modulation of expression level by either of immune inducers tested was found. Similar absence of inducer's effect ($P > 0.05$) was documented in the *in vivo* experiment, where haemocyte RNA was sampled from individual mussels injected with trematode larvae and zymosan (Fig. 2B). Perhaps, the most intriguing observation in this experiment is the high heterogeneity of *M. edulis* population in terms of MytFREP1 and MytFREP2 expression level parity. All of the combinations from lack of one of the genes expression (Fig. 2B; ind. no. 2, 11, 14, 15) to expression of both genes maintained at the same level (Fig. 2B; ind. no. 1, 5, 6, 8, 12, 13) are identified in the small sample of 18 mussels. Additionally, at the preliminary stage of this study and with the same detection method we found the individual which lacked both gene transcripts in haemocytes (not shown).

3.2. EST analysis: seven MytFREP genes are found

The searching in the GenBank EST database revealed copies for described genes and portion of relatively similar sequences from two close-related mytilids, *M. galloprovincialis* [16] and *M. californianus* [unpublished: A. Gracey and colleagues]. Totally, 71 sequences were recovered from the public NCBI EST database and assembled species-specifically using Geneious Pro 4.8.5 software with default parameters. Before assembling, all EST were screened for vector and primer contamination.