Gene 530 (2013) 178–184

Contents lists available at ScienceDirect

Gene

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

Methods paper

Molecular cloning and transcriptional regulation of an allograft inflammatory factor-1 (AIF-1) in Zhikong scallop *Chlamys farreri*

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ARTICLE INFO

Article history:
Accepted 14 August 2013
Available online 31 August 2013

Keywords:
Allograft inflammatory factor-1
Chlamys farreri
Immune response
Bacteria challenge
PAMP stimulation
Larval development

ABSTRACT

The allograft inflammatory factor-1 (AIF-1) is one of the key factors associated with inflammatory response. In the present study, the full-length cDNA of AIF-1 was identified from Zhikong scallop *Chlamys farreri* (named as CfAIF-1) by EST (expressed sequence tag) analysis and RACE (rapid-amplification of cDNA ends) approaches. The cDNA of CfAIF-1 consisted of a 5-terminal untranslated region (UTR) of 58 bp, a 3-UTR of 607 bp with a poly (A) tail, and an open reading frame (ORF) of 468 bp encoding a polypeptide of 155 amino acids with the putative molecular mass of 17.8 kDa. There was an EF hand Ca^{2+}-binding motif in the deduced amino acid sequence of CfAIF-1 which was conserved in other AIF-1s. CfAIF-1 shared closer phylogenetic relationship with invertebrate counterparts than vertebrate. The mRNA transcripts of CfAIF-1 were dominantly expressed in hepatopancreas, hemocytes and adductor. During scallop ontogenesis, the CfAIF-1 mRNA was expressed at a low level at early developmental stages from eggs to blastula, and then increased significantly from gastruta to late veliger larvae ($P < 0.05$). Moreover, the mRNA expression levels of CfAIF-1 in the hemocytes of adult scallop were significantly up-regulated during 12–48 h after LPS, PGN and poly I:C stimulation ($P < 0.01$), but there was no significant fluctuation detected after glucan stimulation. Furthermore, the challenge of bacteria *Vibrio anguillarum* remarkably induced the mRNA expression of CfAIF-1 in hemocytes at 6 h ($P < 0.05$) and 12 h ($P < 0.01$). All these results collectively indicated that CfAIF-1 might be involved in the immune response during the ontogenesis and contribute to the defense against microbe infection in scallops.

1. Introduction

Inflammation is one of the first responses of the immune system to infection, which is initiated upon pathogenic infection (bacterial, viral or parasitic), tissue injury or exogenous agents such as lipopolysaccharides (LPS) (Gouwy et al., 2005; Medzhitov, 2008). In the process of inflammatory, many molecules are recruited, among which a cytokine-responsive macrophage molecule, the allograft inflammatory factor-1 (AIF-1), has recently attracted much attention in both vertebrates and invertebrates.

Members in the AIF family are classified into four different subfamilies based on their molecular weight. AIF-1 is a 17 kDa protein with conserved interferon (IFN)-γ-inducible Ca^{2+} binding EF-hand in cytoplasm (Deininger et al., 2002). It was firstly found in rat and initially demonstrated to be a modulator of the immune response during macrophage activation (Ulrike et al., 1995). Recently, accumulating evidence indicates that AIF-1 plays significant roles in acute and chronic allograft rejections, inflammatory responses, reproductive immunity, immune activation, function of macrophages and even the development of autoimmune diseases in vertebrates (Liu et al., 2007; Tian et al., 2006, 2009; Yang et al., 2005). AIF-1 has been reported to be situated in the putative expressed regions of the major histocompatibility complex (MHC) class III (Watano et al., 2001), where clusters of genes involved in immune responses and cell recognition are located (Iris et al., 1993). The mRNA expressions of AIF-1 in many vertebrates were reported to be significantly up-regulated in response to microbes infection or pattern associated molecular pattern (PAMP) stimulation, indicating its conserved function against external challenge (Miyata et al., 2001; Wang and Wu, 2007). Compared with the knowledge about the multiple functions of AIF-1s in vertebrates, the information about AIF-1 and its involvement in the immune response of invertebrates is still very limited.

The first invertebrate AIF-1 was reported in sponges, and its mRNA expression could be induced in cytokine-mediated allogenic responses during wound repair (Michael et al., 1999). In mollusk, AIF-1s were recently identified in disk abalone (De Zoysa et al., 2010), manila clam (Zhang et al., 2011), pacific oyster (Zhang et al., 2013) and pearl oyster (Li et al., 2013). These AIF-1 molecules were active in the host immune responses against pathogenic challenge, PAMP stimulation or tissue

Abbreviation: AIF-1, allograft inflammatory factor-1; EST, expressed sequence tag; cDNA, complementary deoxyribonucleic acid; RACE, rapid-amplification of cDNA ends; UTR, untranslated region; ORF, open reading frame; PCR, polymerase chain reaction; RT-PCR, real-time PCR; LPS, lipopolysaccharides; PGN, peptidoglycan; MHC, major histocompatibility complex; PAMP, pattern associated molecular pattern; bp, base pair(s); L, liter; min, minute.

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http://dx.doi.org/10.1016/j.gene.2013.08.050

injury in adults (De Zoysa et al., 2010; Zhang et al., 2011). However, the information about their response during early development stages is still needed to better understand functions and the involvements of AIF-1s in the ontogenesis and development of invertebrate immune system.

The scallop *Chlamys farreri* is a bivalve distributed in the coast of China, Korea and Japan. The absence of acquired immunity makes scallops exclusively rely on the innate immune system to protect themselves against continuous threats from pathogens. Considering that inflammation is an important part of innate response, the investigations of the inflammatory-related factors will contribute to further understanding mechanism of the immune defense in scallops. The main objectives of the present study were (1) to clone the full-length cDNA of AIF-1 from *C. farreri* (designated as CfAIF-1), (2) to investigate the tissue-specific expression of CfAIF-1 in adult individuals, (3) to examine the mRNA expression patterns of CfAIF-1 during the scallop ontogenesis and its temporal response profile in hemocytes after PAMPs stimulation or bacterial challenge, and (4) to infer its role in the immune responses of scallop.

2. Materials and methods

2.1. Scallops

Healthy scallops *C. farreri* with an average shell length of 55 mm, were collected from a farm in Qingdao, Shandong Province, China, and maintained in the aerated seawater at 15 °C for a week prior to experimentation.

2.2. RNA isolation and cDNA synthesis

The hemolymph was extracted with a syringe from the adductor muscle and immediately centrifuged at 800 *g*, 4 °C for 10 min to harvest the hemocytes. Total RNA was isolated from the hemocytes of scallops using TRIzol reagent (Invitrogen). The first-strand synthesis was carried out based on Promega M-MLV RT Usage information using the DNase I (Promega)-treated total RNA as template and oligo (dT)-adaptor (5′-GGCCACGCGTCGACTAGTACT$_{17}$-3′) as primer. The reaction mixtures were incubated at 42 °C for 1 h, and terminated by heating at 95 °C for 5 min.

2.3. EST analysis and cloning of the full-length CfAIF cDNA

BLAST analysis of all the EST sequences from a cDNA library of scallop *C. farreri* (Wang et al., 2009) revealed that one EST (no. cl84ct90cn98, 465 bp) was homologous to AIFs from other species. Based on this sequence, two specific primers P1 (5′-GATACAACGGAATCTCAAACGA-3′) and P2 (5′-CGGGTCCAGTATTGGTGTTTCC-3′) were designed to clone the full cDNA sequence of AIF by rapid amplification of cDNA ends (RACE) approach. PCR amplification to clone the 3′ end of CfAIF-1 cDNA was carried out using sense primer P1 and antisense primer T7, while sense primer T3 and antisense primer P2 were used to get the 5′ end. The PCR products were cloned into the pMD18-T simple vector (TaKaRa) and sequenced in both directions with primers M13-47 and RV-MF. The sequencing results were verified and subjected to cluster analysis.

2.4. Sequence analysis

The cDNA sequence and deduced amino acid sequence of CfAIF-1 were analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). The signal peptide was predicted with the Signal P 3.0 server (http://www.cbs.dtu.dk/service/SignalP), and the protein domains were revealed by the simple modular architecture research tool (SMART) version 4.0 (http://www.smart.emblheidelberg.de/). The presumed tertiary structures of CfAIF-1 protein were established using the SWISS-MODEL prediction algorithm (http://swissmodel.expasy.org/) (Arnold et al., 2006; Kiefer et al., 2009) and displayed by PyMOL Viewer 1.3 (http://www.pymol.org). The ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw2/) was used to create the multiple sequence alignment. An unrooted phylogenetic tree was constructed based on the sequence alignment by the neighbor-joining (NJ) algorithm using the MEGA4.1 beta software (http://www.megasoftware.net). The reliability of the branching was tested by bootstrap re-sampling (1000 pseudo-replicates).

2.5. Examination of the temporal expression of CfAIF-1 mRNA in different development stages

All embryos and larvae were sampled from the Yixiang farm in Rongcheng, Shandong Province, China in April and May as described by Yue et al. (2013). The spawning of scallop was induced by thermal shock method. After fertilization, the developing embryos were cultured in filtered and aerated seawater at 20 °C. The embryo or larva at 15 different stages was identified microscopically, including oocytes, fertilized eggs, 2-cell embryos, 4-cell embryos, 8-cell embryos, 16-cell embryos, 32-cell embryos, morula (6 h post-fertilization), blastula (11 h post-fertilization), gastruta (18 h post-fertilization), trochophore larvae (22 h post- fertilization), early D-hinge larvae (48 h post-fertilization), early veliger larvae (96 h post-fertilization), mid-veliger larvae (168 h post-fertilization) and late veliger larvae (552 h post-fertilization). Six duplicate samples collected from each stage were resuspended in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA), and stored in liquid nitrogen immediately. RNA isolation, cDNA synthesis, RT-PCR and statistically analysis were carried out as described above.

2.6. Detection of CfAIF-1 mRNA transcripts in different tissues

The total RNA from hemocytes, gill, adductor muscle, gonad, hepatopancreas and mantle was extracted from six adult scallops as parallel samples using TRIzol reagent (Invitrogen). RNA isolation and cDNA synthesis were carried out as described above. cDNA mix was diluted to 1:100 and stored at −80 °C for subsequent SYBR Green fluorescent quantitative real-time PCR (RT-PCR).

Two CfAIF-1 gene-specific primers, RTF (5′-GGGATGATGTTAGGAGGCAAGA-3′) and RTR (5′-GCCTAGGTGTTACGGCAGAG-3′), were used to amplify a product of 132 bp and the PCR product was sequenced to verify the specificity of RT-PCR. Two β-actin primers, AF (5′-CAAACAGCAGCCTCCTCGT-3′) and AR (5′-CTGGGCACCTGAACCTTTCGTT-3′), were used to amplify a 94 bp fragment as an internal control to verify the successful transcription and to calibrate the cDNA template for corresponding scallop samples. Real-time PCR amplification was carried out in an ABI 7300 Real-time Thermal Cycler according to the manual (Applied Biosystems). Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed using ABI 7300 SDS software version 2.0 (Applied Biosystems). The $2^{-\Delta\Delta CT}$ method was used to analyze the expression level of CfAIF-1 (Kenneth and Thomas, 2001). All data were given in terms of relative mRNA expressed as mean ± S.E. The data were subjected to two-tailed Student's *t* test using SPSS 11.0 software. Difference was considered to be significant at $P < 0.05$.

2.7. Determinations of the temporal pattern of CfAIF-1 mRNA in hemocytes after PAMPs stimulation and Vibrio anguillarum challenge

Three hundred and seventy-five scallops were employed for the PAMPs stimulation experiment and divided into 5 groups randomly. The stimulation was performed by an intramuscular injection of 50 μL phosphate buffered saline (PBS, 0.14 M mol L^{-1} sodium chloride, 3 mmol L^{-1} potassium chloride, 8 mmol L^{-1} disodium hydro-genphosphate dodecahydrate, 1.5 mmol L^{-1} potassium