Fish & Shellfish Immunology 31 (2011) 887–893

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Characterization of a novel molluscan MyD88 family protein from manila clam, *Ruditapes philippinarum*

Youngdeuk Lee [a], Ilson Whang [a], Navaneethaiyer Umasuthan [a], Mahanama De Zoysa [b], Chulhong Oh [c], Do-Hyung Kang [c], Cheol Young Choi [d], Choul-Ji Park [e], Jehee Lee [a,f,*]

[a] Department of Marine Life Sciences, School of Marine Biomedical Sciences, Jeju National University, Jeju Special Self-Governing Province 690-756, Republic of Korea
[b] College of Veterinary Medicine, Chungnam National University, Yuseong-gu, Daejeon 305-764, Republic of Korea
[c] Korea Ocean Research and Development Institute, Ansan 426-744, Republic of Korea
[d] Division of Marine Environment and Bioscience, Korea Maritime University, Busan 606-791, Republic of Korea
[e] Genetics & Breeding Research Center, National Fisheries Research & Development Institute, Geoje 656-842, Republic of Korea
[f] Marine and Environmental Institute, Jeju National University, Jeju Special Self-Governing Province 690-814, Republic of Korea

ARTICLE INFO

Article history:
Received 17 May 2011
Received in revised form
2 August 2011
Accepted 2 August 2011
Available online 10 August 2011

Keywords:
Manila clam
Ruditapes philippinarum
Mollusc
Invertebrate
Myeloid differentiation factor 88 (MyD88)

ABSTRACT

Myeloid differentiation factor 88 (MyD88) is a universal adaptor protein which is required for signal transduction of TLR/IL-1R family. In this study, a novel molluscan MyD88 family member protein (named as RpMyD88) was identified from manila clam, *Ruditapes philippinarum*. It was identified using BLAST algorithm from GS-FLX™ sequencing data. The cDNA of RpMyD88 consists of 1416 bp open reading frame (ORF) encoding 471 amino acid residues. The RpMyD88 contains death domain and Toll/interleukin-1 receptor (TIR) domain which are typical features of MyD88 family proteins. The predicted amino acid sequence of RpMyD88 shares 27% identity with scallop MyD88. The expression level of RpMyD88 mRNA was investigated in healthy and challenged clams by quantitative real-time RT-PCR. The RpMyD88 gene expression is ubiquitous in all selected tissues. The RpMyD88 mRNA was strongly expressed in hemocyte, gill and mantle. In contrast, it was weakly expressed in siphon, foot and adductor muscle. RpMyD88 was up-regulated in gill and hemocyte after immune challenge with both *Vibrio tapetis* and LPS challenge. All results considered, sequence characterization, comparison and gene expression data suggesting that MyD88-dependent signaling pathway is presence in manila clam and RpMyD88 plays an important role in innate immune response against bacteria.

1. Introduction

Innate immunity is evolutionarily ancient, and predates adaptive immunity. As such, it is an organism's first line of defense against invading pathogens [1]. Innate immune responses are triggered when pathogen-associated molecular patterns (PAMPs) come into contact with host-expressed pattern recognition receptors (PRRs) [2,3]. The most well characterized PRRs in vertebrates are the Toll-like receptors (TLRs), which can detect and distinguish PAMPs derived from various microbial pathogens (viruses, bacteria, protozoa and fungi) [4]. These microbial components include bacterial membrane-expressed lipopolysaccharide (LPS), viral RNA, CpG DNA, flagellin, and lipoprotein.

The myeloid differentiation factor 88 (MyD88) is an evolutionarily conserved host-expressed adaptor protein that is essential for proper TLR/Interluekin-1 receptor (IL-1R) immune-response signaling [5]. MyD88 consists of a Toll/IL-1 receptor (TIR) domain, located in the N-terminal region, and a death domain, at its C-terminus. TLRs are known to interact with the TIR domain and be activated through the death domain. MyD88 itself can also interact with the death domains of interleukin-1 receptor associated kinase (IRAK) family members, including IRAK1, IRAK2, IRAK4 and IRAK-M [4,6,7] to trigger downstream signaling cascades that lead to the activation of the transcription factor nuclear factor (NF)-κB.

MyD88 was first identified in 1990 as a protein that was induced during the terminal differentiation of M1D$^+$ myeloid precursors in response to IL-6 [8]. Subsequently, orthologues of this protein have been found in several species, including human [9], mouse [10], frog [11], zebrafish [12], rock bream [13], and fruit fly [14]. However,

* Corresponding author. Department of Marine Life Sciences, School of Marine Biomedical Sciences, Jeju National University, 66 Jejudaehakno, Ara-dong, Jeju Special Self-Governing Province 690-756, Republic of Korea. Tel.: +82 64 754 3472; fax: +82 64 756 3493.
E-mail addresses: jehee@jejunu.ac.kr, jeheedaum@hanmail.net (J. Lee).

1050-4648/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2011.08.003

to our knowledge, molluscan MyD88 has been only reported in scallop [3].

Manila clam, *Ruditapes philippinarum*, is an infaunal bivalve mollusc that is an important constituent of the intertidal zones of the Yellow Sea bordering countries such as Korea and China and Japan [15,16]. Its worldwide distribution as a human food commodity has made it an economically-important species of the Korean aquaculture industry. Recent mass mortalities of manila clam landings, believed to be due to microbial infection, have significantly impacted production and sparked interest in immune-related research of this species [17,18]. The manila clam is known to lack an adaptive immune system, but its mechanisms of innate immunity have yet to be fully elucidated. In contrast, the innate immune system in mammals has been extensively studied and this data may be used to provide significant insights into the immune system used by molluscs. In mammals, the MyD88-dependent TLR signaling pathway relies completely on the presence and proper activity of MyD88 adaptor protein. Therefore, we presumed that MyD88 play a similar role in the immune system of manila clam.

This study was designed to first determine the presence of a MyD88 orthologue in manila clam and subsequently characterize the function of such in response to immune challenge. We describe here our successful cloning of the complete ORF sequence of manila clam MyD88 and the results of comparative analysis with other known MyD88 genes to establish the phylogenetic and evolutionally relationship. In addition, we defined the tissue-specific expression and temporal expression profile in response to immune stimulation (*Vibrio tapetis* and LPS).

2. Materials and methods

2.1. Next-generation sequencing and identification of manila clam MyD88

We have previously established a manila clam cDNA sequence database based upon pyrosequencing data obtained from the 454 Genome Sequencer FLX platform (GS-FLX™; Roche, USA). Briefly, total RNA was isolated from whole body of healthy manila clams using the Trizol reagent (Sigma, USA). Poly(A) mRNA was then isolated using the FastTrack® 2.0 kit (Invitrogen, USA). First-strand cDNA was generated from 1.5 μg of poly(A^+) RNA using a Creator™ SMART™ cDNA library construction kit (Clontech, USA), which was then amplified with the 50X Advantage 2 polymerase mix (Clontech). The resultant cDNA library was normalized using the Trimmer-Direct cDNA normalization kit (Evrogen, Russia). Sequencing of normalized manila clam cDNA was carried out on a GS-FLX Titanium instrument (DNA linker, Korea), and the output reads were processed and assembled by using the Arachne whole-genome shotgun assembler program [19–21]. MyD88 gene from manila clam (designated as RpMyD88) was identified by using the Basic Local Alignment Search Tool (BLAST) algorithm [22].

2.2. Sequence analysis of RpMyD88

The RpMyD88 gene ORF sequence was identified using DNAssist software (Version 2.2; http://www.dnassist.com). To determine the conserved domains of the RpMyD88 predicted protein, motif scan Pfam hidden Markov models (Local models) were used (http://hits.isb-sib.ch/cgi-bin/PFSCAN). Multiple alignment of protein sequences from different species was performed by the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and the identity and similarity percentage was calculated using EMBOSS pairwise alignment algorithms (http://www.ebi.ac.uk/Tools/emboss/align/). The phylogenetic tree was constructed by the Neighbor-Joining method using molecular evolutionary genetic analysis software (MEGA, v5.03; http://www.megasoftware.net).

2.3. Experimental animals and immune challenge

Manila clams, with average shell length of 3.5–4.0 cm, were collected from the Eastern coastal region of Jeju Island (Republic of Korea). In the laboratory, the clams were maintained at 21 ± 1 °C in a flat bottom tank with recirculating seawater. All animals were allowed to acclimatize for one week before any experimentation.

To determine the normal tissue-specific expression of the RpMyD88 gene the following tissues and hemocytes were collected from manila clams: adductor muscle, mantle, siphon, gill and foot. The clam hemolymph was collected from the posterior adductor muscle sinus using 1 ml syringes with 26-gauge needles through the shell hinge. The hemolymph was immediately centrifuged (3500 rpm for 10 min at 4 °C) and the hemocytes were obtained after removal of supernatant.

To evaluate the immune-responsive expression of RpMyD88 gene, whole bacteria (*V. tapetis*, a common manila clam gram-negative pathogen) and purified endotoxin (LPS; *E. coli* 0127:B8; Sigma, USA) were selected for controlled exposure. The *V. tapetis* [KCTC no. 12728] was obtained from the Korean Collection for Type Culture (KCTC). *V. tapetis* and LPS were diluted in phosphate buffer saline (PBS) to make stock. Clams were intramuscularly injected with 100 μL (3.2×10^7 cfu individual^{-1}) of *V. tapetis* and LPS (5 μg individual^{-1}) into adductor muscle. A negative control group was established as un-injected, while PBS control group was injected with an equal volume (100 μL) of PBS. Gill and hemocyte samples were taken from three animals at 3, 6, 12, 24 and 48 h post-challenge. PBS control samples were also isolated at each time point. All samples were immediately snap-frozen in liquid nitrogen and stored at −70 °C until use.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from isolated tissues (50 mg each) using the Trizol reagent (Sigma). First-strand cDNA synthesis was carried out using 1 μg of total RNA as template with the PrimeScript™ first-strand synthesis kit (TaKaRa, Japan) by following the manufacturer's instruction. The cDNA product was diluted 10-fold and stored at −20 °C until further use. Subsequent, quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed to determine tissue-specific expression and expression changes in response to immune stimulation.

2.5. qPCR analysis

Two PCR primers were designed based on the clam MyD88 coding sequence (forward primer, 5′-TGA GCT GGA AGT TAA ACG AGG GCT-3′; reverse primer, 5′-TCA CCA CTC TAC GGC ATC TTG CTT-3′). Two internal control primers were synthesized to amplify β-actin (forward primer, 5′-CTC CCT TGA GAA GAG CTA CGA-3′; reverse primer, 5′-GAT ACC AGC AGA TTC CAT ACC C-3′) [23,24]. qPCR was performed in the Thermal Cycler Dice real-time system (TP800; TaKaRa, Japan) under the following conditions: one denaturation cycle of 95 °C for 3 min, followed by 45 amplification cycles of 95 °C for 20 s, 58 °C for 20 s, 72 °C for 30 s. The baseline was set automatically by the accompanying system software (version 2.0). The relative expression of each gene was determined by the Livak ($2^{-\Delta\Delta CT}$) method [25]. For tissue-specific expression analysis, relative expression of RpMyD88 mRNA was normalized to the expression detected in healthy adductor muscle samples. To determine expression fold-change after immune challenge, the