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Scavenger receptor B promotes bacteria clearance by enhancing phagocytosis and attenuates white spot syndrome virus proliferation in *Scylla paramamosian*

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ABSTRACT

Phagocytosis and apoptosis are key cellular innate immune responses against bacteria and virus in invertebrates. Class B scavenger receptors (SRBs), which contain a CD36 domain, are critical pattern recognition receptors (PRRs) of phagocytosis for bacteria and apoptotic cells. In the present study, we identified a member of SRB subfamily in mud crab *Scylla paramamosain*, named *Sp*-SRB. The full-length cDNA of *Sp*-SRB is 2593 bp with a 1629 bp open reading frame (ORF) encoding a putative protein of 542 amino acids, and predicted to contain a CD36 domain with two transmembrane regions at the C- and N-terminals. Real-time qPCR analysis revealed that *Sp*-SRB was widely expressed in all tissues tested, and the expression of *Sp*-SRB was up-regulated upon challenge with *Vibrio parahaemolyticus*, white spot syndrome virus (WSSV), lipopolysaccharides (LPS) and polyinosinic polycytidylic acid (PolyI:C). Moreover, *in vitro* experiments indicated that recombinant *Sp*-SRB protein (*rSp*-SRB) could bind to fungi, Gram-positive and Gram-negative bacteria. RNA interference of *Sp*-SRB resulted in significant reduction in the expression level of phagocytosis related genes, antimicrobial peptides (AMPs) and Toll-like receptors (TLRs), which consequently led to impairment in both bacterial clearance and the phagocytotic activity of hemocytes. In addition, we found that *Sp*-SRB had the ability to attenuate the replication of WSSV proliferation in mud crab *S. paramamosain*. Collectively, this study has shown that *Sp*-SRB contributed to bacteria clearance by enhancing phagocytosis and up-regulating the expression of AMPs possibly in a TRLs (*Sp*Toll 1 and *Sp*Toll 2)-dependent manner. Besides, *Sp*-SRB inhibited the replication of WSSV in *S. paramamosian* probably through enhancement of hemocytes phagocytosis of apoptotic cells.

1. Introduction

Scylla paramamosain is an important economically mud crab widely distributed in China and Indo-west Pacific countries. In China, the gross production of mud crab would reach a high of 140,000 tons and more than 10 billion RMB yuan annually. Meanwhile, arthropods including mud crab, usually use humoral and cellular defenses to protect themselves from pathogenic microbes [1]. Phagocytosis and apoptosis are key cellular innate immune responses [2,3]. In insects, phagocytosis plays a vital role in innate immunity defense [4]. For instance, in *Drosophila* larvae, phagocytosis mediated by blood cells is the primary defense against disease agents [5]. Phagocytosis is a crucial process in development, tissue homeostasis and immunity, and particles recognition and ingestion [6]. Recognition of a particle or ligand by pattern recognition receptors (PRRs) initiates the phagocytic process [5,7].

Scavenger receptors (SRs), one of the subfamilies of the PRRs, recognize a wide variety of ligands, such as danger-associated molecular patterns (DAMPs), including modified lipoproteins, and various types of exogenous ligands [8]. Based on their protein domain architecture, SRs are divided into eight different classes (A-H), including Class A (SR-A1, MARCO, SRCL), Class B (SCARB1, CD36), Class C (dSR-CI), Class D (CD 68), Class E (LOX1), Class F (SCARF1), Class G (CXCL16), and Class H (Stabilin-1, Stabilin-2) [9]. SR-A, the class A scavenger receptor, was firstly cloned, and discovered for its ability to bind acetylated low-density lipoprotein (AcLDL) [10]. Class C scavenger receptor was only discovered in invertebrates, such as dSR-CI in *Drosophila melanogaster* and *Mj*SRC in *Marsupenaeus japonicus* [11]. Scavenger receptors could bind both modified and unmodified endogenous ligands, but only the class B receptors (CD36 and SR-BI) have been proved to bind unmodified lipoproteins [12]. Class B scavenger receptors (SRBs) contain

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a CD36 domain, with CD36 being an integral transmembrane glycoprotein with two transmembrane domains, a large extracellular loop containing multiple glycosylation sites, and two short intracellular tails [8,13]. In response to the challenge of Lipoteichoic acid (LTA) or diacylated lipoproteins, CD36 has been demonstrated to form complexes with the Toll-like receptors 2 (TLR2)/TLR6 heterodimer and then induces inflammatory reactions in mammalian immunity [14]. CD36 also cooperates with a TLR4/6 heterodimer reaction in sterile inflammation mediated by oxidized low-density lipoprotein (OxLDL) [15]. The monocytic CD36 contributes to the non-opsonic phagocytosis of Plasmodium falciparum-parasitized erythrocytes (PEs) using both the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinases (MAPK) signaling cascades [16]. Another study has shown that as a phagocytic receptor for a variety of microbes, CD36 mediates signaling induced by LPS and Gram-negative bacteria via a Jun N-terminal Kinase (JNK) mediated signaling pathway in a TLR2/4-independent manner [17]. Besides, as a phagocytic receptor and a modulator of the early innate immune response, CD36 contributes to the pulmonary host response during *Streptococcus pneumoniae* infection in mice [18]. Research on *Drosophila* shows that Croquemort (Crq), a *Drosophila* member of the CD36 family, is required in phagocytes for efficient uptake of bacteria and fungi [19].

In marine invertebrates, several SRB genes have been discovered. *Mj*Croquemort, a Croquemort homology, was identified in *Marsupenaeus japonicas* [20]. Following this, *Mj*SR-B1, a novel member of the SR-B family, in *M. japonicus* was reported to be a phagocytic receptor which can bind to both Gram-positive and Gram-negative bacteria and regulate the expression of AMPs [21]. Besides, *Mn*SR-BI, a member of the CD36 superfamily, was reported in *Macrobrachium nipponense* [22]. However, little is known about SRB in mud crab. In the present study, we identified a member of the SRB subfamily in mud crab *S. paramamosain*, named *Sp*-SRB. We found that *Sp*-SRB had a CD36 domain with two transmembrane regions at its N- and C-terminals.

The expression of *Sp*-SRB in mud crab was upregulated after challenging with *V. parahaemolyticus,* WSSV, LPS or PolyI:C. Moreover, *in vitro* experiments revealed that the recombinant protein, *rSp*-SRB, had different binding abilities to fungi, Gram-positive and Gram-negative bacteria. After *Sp*-SRB knockdown, the expression level of phagocytosis related genes, AMPs and TRLs (*Sp*Toll 1 and *Sp*Toll 2) decreased, along with a reduction in the clearance of bacteria and phagocytotic rate of hemocytes. In addition, we demonstrated that *Sp*-SRB could restrict the proliferation of WSSV in mud crab *S. paramamosain*.

2. Materials and methods

2.1. Immune challenge and tissue collection

Healthy mud crabs (approximately 100 g each) were purchased from a crab farm in Niutianyang (Shantou, Guangdong, China), and acclimated in laboratory tanks in 10‰ salinity water at 25 °C for a week before further processing. For challenge experiments, 200 μl *V. parahaemolyticus* (1×10^7 cfu/ml) [23], WSSV (1×10^6 virus copies/ml), LPS (0.5 mg/ml), or PolyI:C (1 mg/ml, Sigma, USA), was injected into the base of the fourth leg of each crab. For blank control, 200 μl 0.8% NaCl (normal saline, NS) (Sangon Biotech, Shanghai, China) was used. Hemolymph was withdrawn from three randomly chosen crabs per group after anesthetizing with ice at 0, 6, 12, 24, 48, and 96 h post injection (hpi), and collected into tubes containing ice-cold acid citrate dextrose (ACD) anticoagulant buffer (1.32% sodium citrate, 0.48% citric acid,1.47% glucose), then immediately centrifuged at 800 × g for 10 min at 4 °C to separate the hemocytes, which was then used for RNA extraction with TRIzol® Reagent (Ambion, USA). Other tissues including stomach, muscle, subcuticular epidermis, gills, hepatopancreas, intestines, and heart of *S. paramamosain* were quickly collected, and immediately dipped into liquid nitrogen for later use. Tissues were collected from at least three mud crabs for subsequent total RNA extraction.

2.2. Gene cloning and bioinformatics analysis

A total of 5 μg RNA (from hemocytes) was reverse transcribed with M-MLV First-Strand cDNA Synthesis Kit (Invitrogen, USA). Partial cDNA sequence of *Sp*-SRB was obtained from our previous high-throughput transcriptome data. The complete cDNA was amplified via 3′RACE and 5′RACE PCR with the SMARTer™ RACE cDNA Amplification Kit (Clontech, USA), using touch-down PCR and nested PCR strategy with specific primers (Table 1). The product of touch-down PCR was used as template for the subsequent nested PCR. Purified DNA fragments were cloned into the pMD® 19-T vector (TaKaRa, Dalian, China) and then transformed into *Escherichia coli*. Positive recombinant clones were identified by PCR screening with M13R and M13F primers, and sequenced by a commercial company (BGI, Shenzhen, China).

The deduced amino acid sequence was obtained with the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). Further, the 3D structure of *Sp*-SRB was predicted by SWISS-MODEL (http://swissmodel.expasy.org/), while MEGA 5.10 was used to construct phylogenetic trees.

2.3. Real-time quantitative PCR assay

Total RNA was extracted from tissues followed by first-strand cDNA synthesis using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Two gene-specific primers (Q-*Sp*-SRBF and Q-*Sp*-SRBR, Table 1) were synthesized to amplify *Sp*-SRB with β-actin as the internal control. The real-time quantitative PCR (qPCR) was carried out using the TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) in LightCycler ® 480 (Roche, USA). The amplification procedure included a denaturation step of 95 °C for 30 s, and 40 cycles of 95 °C for 5 s, 60 °C for 20 s, followed by a melting curve analysis from 65 °C to 95 °C. Each sample was in triplicate. The PCR data was analyzed using the LightCycler 480 software (Roche, USA). Relative transcript level of *Sp*-SRB was determined using the $2^{-\Delta\Delta Ct}$ algorithm. The data were subjected to one-way ANOVA analysis using Origin Pro8.0 followed by *t*-test, with $P < 0.05$ considered statistically significant.

2.4. Expression and purification of recombinant Sp-SRB

Specific primers (*rSp*-SRBF and *rSp*-SRBR, Table 1) were used to amplify the fragment that encodes the extracellular region of *Sp*-SRB. After digestion with *Eco*R I and *Xho* I, the PCR products were cloned into pGEX-4T-1 and transformed into Rosetta-gami™2 (DE3) plysS competent cells (Novagen, Germany) for protein expression. After IPTG induction, the recombination protein expressed in the supernatant was purified as follows: harvested cells were re-suspended in TBS (50 mM Tris, 100 mM NaCl, pH 8.0) plus 10 mM dithiothreitol (DTT), and sonicated at 4 °C for 25 min with a sonicator (BILON-250Y) set at 3 s sonication and 4 s interval under 35% power. Next, the cell lysates were centrifuged at 10,000 rpm for 15 min at 4 °C to collect the supernatant (containing GST), and then ProteinIso ® GST Resin (TransGen Biotech, Beijing, China) was added to purify the proteins.

2.5. Bacterial and fungal binding assay and western blotting

Cultures of the fungus *Saccharomyces cerevisiae,* Gram-positive bacteria *Staphylococcus aureus* and *Beta streptococcus*, Gram-negative bacteria *V. parahaemolyticus*, *Vibrio alginolyticus*, *Aeromonas hydrophila* and *E. coli,* were centrifuged and washed three times with 1 ml TBS, and suspended in TBS buffer at 2×10^8 CFU/ml. Next, 500 μl of each bacterium or fungus was incubated with 500 μl (100 μg/ml) of *rSp*-SRB for 1 h at room temperature with gentle rotation. After that, the bacteria or