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Function of gC1qR in innate immunity of Chinese mitten crab, *Eriocheir sinensis*

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ABSTRACT

gC1qR is identified as the globular "head" binding protein of the C1q protein and performs an important function in innate immunity. A *EsgC1qR* gene was identified from the hepatopancreas of *Eriocheir sinensis*. *EsgC1qR* encodes a protein with 275 amino acids. Phylogenetic analysis showed that, together with crustaceans gC1qRs, EsgC1qR belongs to one group. *EsgC1qR* mRNA was detected in hemocytes, intestine, hepatopancreas, gills, eyestalk, heart, muscle, and nerve. The expression of the *EsgC1qR* transcript in the hepatopancreas could be regulated by lipopolysaccharides (LPS), peptidoglycans (PGN), *Staphyloccocus aureus*, or *Vibrio parahaemolyticus*. Recombinant EsgC1qR (rEsgC1qR) protein could bind to various bacteria, LPS, and PGN. rEsgC1qR protein also presents direct bacteria inhibitory activity. rEsgC1qR could interact with EsCnx or EsCrt. Therefore, from the results, we could speculate that EsgC1qR is involved in the innate immunity of Chinese mitten crab, *E. sinensis*.

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1. Introduction

The Chinese mitten crab, *Eriocheir sinensis*, is an economically important species that is cultured in China and other Asian countries (Li et al., 2007). In recent years, its production sharply declined because of serious infectious diseases caused by viruses, fungi, bacteria, spiroplasma, and parasites (Bonami and Zhang, 2011; Morado, 2011; Wang, 2011). Owing to the lack of a true adaptive immune system, crabs depend entirely on the non-specific innate immune system for protection against pathogen invasion (Hoffmann and Reichhart, 2002; Mu et al., 2011).

The complement system performs a critical function in host defense and inflammation. Complement activation results in opsonization of pathogens and their removal by phagocytes, as well as cell lysis (Walport, 2001a,b; Dunkelberger and Song, 2010; Sarma and Ward, 2010). The complement system consists of a tightly regulated network of proteins and is initially triggered by the recognition of different substrates by complement component 1 (C1), lectin, or C3 tick-over (Walport, 2001a, 2001b; Janeway et al., 2005). The C1 complex, which consists of C1r, C1s, and C1q, performs a crucial function in the induction of primary immune response (Nicholson-Weller and Klickstein, 1999). As a subunit of the first component of complement, C1q contains two major structural and functional domains, namely, collagen-like "stalk" (cC1q) and globular "heads" (gC1q) (Peerschke and Ghebrehiwet, 2014). Two glycoproteins have been identified to bind to cC1q or gC1q and thus designated as cC1qR and gC1qR, respectively (Ghebrehiwet and Peerschke, 2004).

gC1qR, which is also known as p33, complement component 1q subcomponent binding protein (C1qBP), and HABP1, is a highly anionic cellular protein with affinity to various ligands, such as the globular head of C1q, high-molecular-weight kininogen, hyaluronic acid, and fibronectin (Ghebrehiwet et al., 1994, 2001; Ghebrehiwet and Peerschke, 1998; Braun et al., 2000). gC1qR is a single-chain protein with an apparent molecular mass of 33 kDa and is a ubiquitously expressed, biologically important, multifunctional, and multicompartmental protein (Peerschke et al., 1994; Yao et al., 2004; Xu et al., 2009). gC1qR exists in numerous compartments of a cell, including nucleus, cytoplasm, mitochondria, plasma membrane, and cell surface (Ghebrehiwet et al., 2001; Fogal et al., 2008). gC1qR expressed on the cell surface can serve as a receptor for numerous extracellular and intracellular, microbial, and viral proteins, and was originally called a multifunctional chaperone (Lim et al., 1996; Nguyen et al., 2000; Yao et al., 2004).

Recently, an increasing number of reports have demonstrated that gC1qR may participate in the innate immune system of

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vertebrates (Peerschke and Ghebrehiwet, 2007). Several studies focusing on invertebrate gC1qR could also be obtained. A gC1qR in *Fenneropenaeus chinensis* may be involved in anti-bacterial and anti-viral infection (Li et al., 2012). The C1qBP from *Penaeus monodon* (PmC1qBP) could intersect with mouse C1q (Yang et al., 2013). A gC1qR function as a pathogen–recognition receptor (PRR) was identified from *Macrobrachium rosenbergii* (Ye et al., 2015). Information on the biological functions of gC1qR in crab immunity is lacking. Therefore, in this study, we mainly focused on the function of crab gC1qR in innate immunity.

In this study, a *EsgC1qR* gene was identified and characterized in Chinese mitten crab, *E. sinensis*. Its mRNA expression pattern upon LPS, PGN, and bacteria challenge was studied. The microorganisms and polysaccharides binding activity, bacteria inhibitory activity, and interaction with calreticulin or calnexin were analyzed in vitro. These results indicate the probable functions of *EsgC1qR* in crab innate immunity.

2. Materials and methods

2.1. Crabs, tissue collection, and microorganism challenges

E. sinensis, averaging 60 g ± 5 g in weight were obtained from an aquatic market in Nanjing, Jiangsu Province, China, and maintained in filtered aerated freshwater at 20–25 °C for a week before processing. Various tissues (hemocytes, heart, hepatopancreas, gills, muscle, intestine, nerve, and eyestalk from five adult crabs as parallel samples) were collected to determine the tissue distribution of *EsgC1qR* transcript. Hemolymph was collected by using a syringe with an equal volume of anticoagulant solution (ACD-B) (Huang et al., 2013). Samples were immediately centrifuged at 800*g* and 4 °C for 15 min to harvest hemocytes. A total of 150 crabs were used for stimulation experiment and randomly divided into five groups (30 individuals in each group). Four groups of crabs were injected with 50 μL LPS, (0.5 μg/μL), PGN (0.5 μg/μL), *Staphyloccocus aureus* (3×10^7 cells), or *Vibrio parahaemolyticus* (3×10^7 cells) in phosphate-buffered saline (PBS) respectively according to our previous description (Huang et al., 2014), and five individual challenged crabs from each group were randomly collected at 2, 6, 12, and 24 h post-treatment. Untreated crabs were used as the blank group. Hepatopancreas was collected from untreated control and microorganism challenged (LPS, PGN, *S. aureus*, and *V. parahaemolyticus*) treatment groups. All tissue samples were stored at −80 °C for subsequent RNA extraction.

2.2. Total RNA isolation and cDNA synthesis

The total RNA was extracted from various tissues (hemocytes, heart, hepatopancreas, gills, muscle, intestine, nerve, and eyestalk) by following the manufacturer's instructions (Spin-column, Bio-Teke, Beijing, China). The integrity of total RNA was routinely checked with 1% agarose gel electrophoresis. The synthesis of first-strand cDNA was carried out using PrimeScript® 1st Strand cDNA synthesis kit with Oligo dT Primer from Takara (Dalian, China). The cDNA mix was incubated at 30 °C for 10 min and 42 °C for 1 h, terminated by heating at 72 °C for 15 min, diluted to 1:10 by the diethyl pyrocarbonate DEPC treated water, and then stored at −80 °C for subsequent reverse transcriptase quantitative PCR.

2.3. Cloning of full-length cDNAs of EsgC1qR

A cDNA library was constructed from the hepatopancreas of *E. sinensis*. BLAST analysis indicated that one expressed sequence tag (EST) is homologous to previously identified gC1qR and assembled into one unigene. Based on the obtained cDNA sequence of *EsgC1qR*, two gene-specific primers (EsgC1qR-F: 5′-GCGAGGAGGCTGGGGAAGATGTGTTT-3′, EsgC1qR-R: 5′-TGCCAAACACATCTTCCCCAGCCTCC-3′) were designed to clone the full-length sequence of *EsgC1qR*.

2.4. Sequence analysis

The cDNA sequences and deduced amino acid sequences of EsgC1qR were analyzed by using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Expert Protein Analysis System (http://web.expasy.org/translate/). Protein domains were predicted with a simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/). Homology analysis was conducted by using the Ident and Sim Analysis provided on http://www.bioinformatics.org/sms/. Calculated EsgC1qR molecular weight and predicted isoelectric point were determined through ExPASy (http://web.expasy.org/compute_pi/). A phylogenetic tree was constructed based on the deduced amino acid sequences of EsgC1qR and other known gC1qR by the neighbor-joining (NJ) algorithm by using the MEGA 5.05 software (Kumar et al., 2008). For deriving the confidence value for phylogeny analysis, bootstrap trials were replicated for 1000 times. MEGA 5.05 and GENDOC were also used to create the multiple sequence alignments.

2.5. Quantitative analysis of EsgC1qR mRNA expression

Two gene-specific primers for *EsgC1qR*, EsgC1qR-RT-F (5′-CTTACAGTTTATGAGGGCGAGTGGA-3′) and EsgC1qR-RT-R (5′-GGGAGTGTTCGTAGTCGGAGCA-3′), were used to amplify a product of 166 bp from cDNA, and the PCR product was sequenced to verify the specificity of qRT-PCR. Two glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, EsGAPDH-RT-F (5′-CTGCCCAAAACATCATCCCATC-3′) and EsGAPDH-RT-R (5′-CTCTCATCCCCAGTGAAATCGC-3′), were used to amplify a 268 bp fragment as an internal control to verify successful transcription and calibrate the cDNA template for corresponding samples. DEPC–water for the replacement of cDNA template was used as negative control. All samples were repeated in triplicate in qRT-PCR analysis. Data were calculated using $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001) and subjected to statistical analysis. Unpaired sample *t*-test was conducted, and differences were considered significant if $P < 0.05$.

2.6. Expression and purification of recombinant EsgC1qR

A pair of primers, namely, EsgC1qR-ex-F (5′-TACTCAGGATCCATGCTGTGCTCCTGCGGCTGC-3′) and EsgC1qR-ex-R (5′-TACTCAGCGGCCGCCTTGCGCTTGACGAAATCCTG-3′), with *BamH* I and *Not* I sites in the forward and reverse primers were used to amplify the fragment encoding the MAM33 domain of EsgC1qR. Both fragment and pET30a (+) were digested with the corresponding restriction enzymes and ligated. The cDNA fragments were inserted into the pET30a (+) plasmid. The recombinant plasmid pET30a–EsgC1qR was transformed into competent *Escherichia coli* BL21 (DE3) cells for isopropyl-b-D-1-thiogalactopyranoside (IPTG)-induced recombinant expression. The recombinant EsgC1qR was purified by His Bind resin chromatography (Novagen, USA) according to the manufacturer's instruction.

2.7. Microorganism binding assay

Eight species of microorganisms were used for the assay. These microorganisms include Gram-positive (*S. aureus*, *Micrococcus luteus*, *Bacillus subtilis*, and *Bacillus megaterium*) and Gram-negative bacteria (*Aeromonas hydrophila*, *V. parahaemolyticus*, *Vibrio*

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