Developmental and Comparative Immunology 78 (2018) 42–51

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Comparative study of three C1q domain containing proteins from pacific oyster *Crassostrea gigas*

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ARTICLE INFO

Article history:
Received 5 February 2017
Received in revised form
23 June 2017
Accepted 14 September 2017
Available online 18 September 2017

Keywords:
C1qDC proteins
LPS binding affinity
Gram-negative bacteria
Recognition specificity

ABSTRACT

C1q domain containing proteins (C1qDCs) are a family of proteins containing a globular head C1q domain (ghC1q) in C-terminus, which serve as pattern recognition receptors (PRRs) and mediate a series of immune responses. In the present study, three C1qDC proteins from pacific oyster *Crassostrea gigas* (*Cg*C1qDC-2, *Cg*C1qDC-3, *Cg*C1qDC-4) were characterized and comparatively investigated to understand their roles in the immune response. All the three recombinant *Cg*C1qDC proteins (r*Cg*C1qDCs) could bind lipopolysaccharide (LPS) significantly but they could not bind lipoteichoic acid (LTA), β-1,3-glucan (GLU), mannan (MAN), and polyinosinic-polycytidylic acid (Poly I:C). Correspondingly, they all exhibited higher binding activities towards Gram-negative bacteria *Vibrio anguillarum* and *V. splendidus*. Moreover, they could enhance the phagocytosis of oyster hemocytes, and the enhancements towards Gram-negative bacteria were significantly higher than that towards Gram-positive bacteria ($p < 0.01$). The LPS binding affinity of r*Cg*C1qDC-3 ($K_D = 8.74 \times 10^{-7}$ M) was higher than that of r*Cg*C1qDC-2 ($K_D = 7.76 \times 10^{-5}$ M) and r*Cg*C1qDC-4 ($K_D = 1.09 \times 10^{-5}$ M). Meanwhile, r*Cg*C1qDC-3 exhibited significantly higher enhancement on phagocytosis of oyster hemocytes towards Gram-negative bacteria than that of r*Cg*C1qDC-2 and r*Cg*C1qDC-4 ($p < 0.05$). After the secondary challenge with *V. splendidus*, the up-regulations of *CgC1qDC-2* and *CgC1qDC-4* mRNA in hemocytes occurred at 6 h, while that of *CgC1qDC-3* was observed at 3 h and lasted for 24 h. And *CgC1qDC-3* responded with high mRNA level for tested 24 h upon the secondary challenge with *V. anguillarum* as well. These results collectively suggested that three *Cg*C1qDCs could serve as PRRs to specifically recognize certain Gram-negative bacteria and opsonins to enhance phagocytosis. *Cg*C1qDC-3, with higher binding affinity to LPS, stronger opsonization and more rapid and persistent mRNA expression response upon the secondary challenge with homologous *Vibrios*, might exert efficient functions in the immune responses against invading pathogens.

1. Introduction

Invertebrates without B/T lymphocyte receptors and functional antibodies are commonly considered lack adaptive immunity (immune memory and immune specificity), and they entirely rely on innate immunity to withstand pathogens (Hauton and Smith, 2007; Turvey and Broide, 2010). Pattern recognition receptors (PRRs) encoded by germline genes are immune recognition molecules to sense the pathogen-associated molecular patterns (PAMPs), and mediate downstream immune responses, playing vital roles in innate immunity (Cao, 2016).

In the past decades, expanded PRR genes have been identified in invertebrates (Dishaw et al., 2012; Huang et al., 2008; Rast et al., 2006; Zhang et al., 2012), and the PRRs in invertebrates are amazingly more abundant than those in vertebrates. Recently, some PRRs with highly genetic diversity were found to promisingly exhibit recognition specificity, helping to target more foreign pathogens for invertebrates. The FREP proteins in *Biomphalaria*

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http://dx.doi.org/10.1016/j.dci.2017.09.014
0145-305X/

glabrata are highly variable, and they can produce mutants involved in more immune recognitions through somatic hypermutation (Zhang and Loker, 2003). In *Drosophila* and *Anopheles mosquito*, the Down syndrome cell adhesion molecules (Dscams) with immunoglobulin domains (Ig domains) could produce more than 30,000 gene subtypes through the exon alternative splicing (Kurtz and Armitage, 2006). The RAG transposon was discovered and the existence of V (D) J recombination was firstly proclaimed in amphioxus, which might provide the mechanism basis for high diversity of antigen receptors in immune specificity of invertebrates (Huang et al., 2016). In mammals, the antibody was selected with high-affinity to a particular antigen in the germinal center, endowing efficiently specific immune responses (Gitlin et al., 2014). In invertebrates, PRRs with large number of family members, high genetic diversity, and high-affinity to specific antigen are most likely to be one of the molecular basis of immune specificity. It is a very interesting topic how the numerous PRRs exert functional divergence and mediate different immune responses in invertebrates (Ward and Rosenthal, 2014).

The C1qDC proteins are a huge family of proteins containing a ghC1q domain of around 140 amino acids in the C-terminus (Kishore and Reid, 2000). C1qDCs in invertebrates are mainly characterized as PRRs to trigger downstream immune responses (Carland and Gerwick, 2010). They could bind a variety of ligands from virus, bacteria, apoptotic cell, and β-amyliod fibers, involving in biological processes including pathogens or apoptotic cells clearance, cell phagocytosis, cell chemotaxis, cell adhesion, inflammatory and oxidative stress response (Ghai et al., 2007). And many C1qDCs were found to exert diverse biological functions in invertebrate immunity. For instance, *Mg*C1q in *Mytilus galloprovincialis* hemocytes drastically responded to the stimulations of Gram-positive bacteria and Gram-negative bacteria at mRNA expression level (Gerdol et al., 2011). *Ai*C1qDC-1 from *Argopecten irradians* was found to agglutinate fungi cells (Kong et al., 2010; Wang et al., 2012a). *Cf*C1qDC from *Chlamys farreri* could bind various PAMPs (LPS, PGN, β-glucan, Poly I: C), and human heat-aggregating IgG (Wang et al., 2012b; Zhang et al., 2008). To date, C1qDC proteins in invertebrates were identified to play an important role in innate immunity, but their functional divergence and recognition specificity to pathogens are largely unknown.

Pacific oyster, *C. gigas*, is one of the most important cultured mollusks worldwide, and the recent released whole genome sequence of pacific oyster was helpful for exploring the molecular mechanisms of molluscan immunity (Zhang et al., 2012). C1qDC gene family in oyster *C. gigas* is very large with 321 annotated genes, which could be promising molecules involved in immune specificity with functional divergence. And it is previously found that *Cg*C1qDC-1 from *C. gigas* with high LPS binding affinity served as a PRR and opsonin (Jiang et al., 2015). In the present study, three single ghC1q domain proteins from pacific oyster *C. gigas* (*Cg*C1qDC-2, *Cg*C1qDC-3, *Cg*C1qDC-4) were comparatively studied to, (1) find their binding specificity to bacteria and PAMPs; (2) compare their difference in LPS binding affinity and involvements in the process of enhancing phagocytosis; (3) monitor their difference of mRNA expression patterns in response to the secondary challenge with homologous *Vibrios*, aiming to provide possible evidence for that PRRs could be involved in immune specificity through functional divergence in invertebrates.

2. Materials and methods

2.1. Experimental animals and microbes

Pacific oysters (*C. gigas*), about 12 cm in length and 180 g in weight, were collected from an aquaculture farm in Qingdao, China and cultured in aerated water tank for a week prior to use. Female mice were provided by the Qingdao Institute of Drug Control (Qingdao, China). Gram-negative bacteria *V. splendidus* and *V. anguillarum* were grown in 2216E medium at 18 °C and 28 °C for 24 h, respectively. Gram-positive bacteria *S. aureus* and *M. luteus* were cultured in LB medium at 37 °C for 12 h.

2.2. Sequential challenge with Vibrios and sample collection

After temporarily cultured for seven days, one hundred and twenty oysters were divided into two groups, which received primary stimulation on adductor muscle with 100 μL of sterilized seawater (designated as s group) or 100 μL of formalin-fixed *V. splendidus* at 2×10^8 CFU mL^{-1} (v group), respectively. Seven days later, oysters from s group and v group were divided into four sub-groups, which received the secondary challenge with 100 μL of sterilized seawater (ss, vs group) or 100 μL of living *V. splendidus* at 2×10^8 CFU mL^{-1} (sv, vv group), respectively. Six oysters were randomly sampled in each experimental group at 0, 3, 6, 12, and 24 h after secondary challenge, and the haemolymph with the constant volume of 1 mL was collected from each oyster. The hemocytes from an oyster (10^6 ~ 10^7 cells) were harvested by centrifugation at 800*g*, 4 °C for 10 min for RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR).

The cross immune challenge with *V. splendidus and V. anguillarum* was conducted as the description above with some modifications and specific treatment information was shown as Supplementary Fig. 1. In brief, two hundred and forty oysters were equally distributed into three groups. Oysters received primary stimulation on adductor muscle with 100 μL of sterilized seawater, 100 μL of formalin-fixed *V. splendidus* (2×10^8 CFU mL^{-1}), or 100 μL of formalin-fixed *V. anguillarum* (2×10^8 CFU mL^{-1}), were designated as sw group, vs group, va group, respectively. Seven days later, oysters from sw, vs, va group were further divided into six sub-groups of sw-vs, sw-va, vs-vs, vs-va, va-vs, va-va receiving secondary challenge with 100 μL of living *V. splendidus* (2×10^8 CFU mL^{-1}) or 100 μL of living *V. anguillarum* (2×10^8 CFU mL^{-1}), respectively. The hemocyte samples from each experimental group were randomly obtained at 0, 6, 12, and 24 h after secondary challenge (N = 6).

The samples for detecting tissue distribution of mRNAs were collected from gill, mantle, adductor muscle, hepatopancreas, gonad and hemolymph of oysters. And all samples above were stored at −80 °C.

2.3. RNA extraction, cDNA synthesis and full-length cDNA cloning

Total RNA from samples was extracted by Trizol reagent (Invitrogen), and DNase I (Promega) was used to digest genomic DNA. The first strand cDNA was synthesized by using M-MLV reverse transcriptase (Promega) according to the manufacturer's information. Then cDNA was synthesized as templates and stored at −80 °C.

The recombinant protein of a phagocytic receptor Integrin (r*Cg*Integrin) in pacific oyster *C. gigas* (Jia et al., 2015), was used as the bait protein and pull-down assay was conducted to obtain the prey proteins from oyster serum. Prey protein bands were predicted as C1qDC proteins by mass spectrometry analysis. Three C1qDCs were identified in the present study, which were designed as *Cg*C1qDC-2, *Cg*C1qDC-3, and *Cg*C1qDC-4. The full-length cDNA sequences of *CgC1qDC-2* (CGI_10001483), *CgC1qDC-3* (CGI_10011387), and *CgC1qDC-4* (CGI_10015754) in oyster were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/), and amplified by using ExTaq DNA polymerase (Takara). All the information of cloning primers used in this study was listed in Table 1. The PCR products were inserted into pMD19-T simple vector (Takara), and