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The sequence variation and functional differentiation of CRDs in a scallop multiple CRDs containing lectin

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ABSTRACT

A C-type lectin of multiple CRDs (CfLec-4) from *Chlamys farreri* was selected to investigate the sequence variation and functional differentiation of its CRDs. Its four CRDs with EPD/LSD, EPN/FAD, EPN/LND and EPN/YND key motifs were recombined separately. The recombinant proteins of CRD1 and CRD2 (designated as rCRD1 and rCRD2) could bind LPS and mannan, while the recombinant proteins of CRD3 and CRD4 (designated as rCRD3 and rCRD4) could bind LPS, PGN, mannan and glucan. Moreover, rCRD3 displayed broad microbe binding spectrum towards Gram-positive bacteria *Staphylococcus aureus* and *Micrococcus luteus*, Gram-negative bacteria *Escherichia coli* and *Vibrio anguillarum*, as well as fungi *Pichia pastoris* and *Yarrowia lipolytica*. These results indicated CRD3 contributed more to CfLec-4's nonself-recognition ability. Furthermore, CRD1, CRD3 and CRD4 functioned as opsonin participating in the clearance against invaders in scallops. The sequence variation in Ca^{2+} binding site 2 among CRDs was suspected to be associated with such functional differentiation.

1. Introduction

C-type lectins are a superfamily of Ca^{2+}-dependent carbohydrate-recognition proteins. They function as PRR to discriminate self and nonself by recognizing and binding the terminal sugars from various microbes (Weis et al., 1998; Zelensky and Gready, 2005). The carbohydrate recognition domains (CRDs) endow C-type lectins with activities of nonself-recognition and clearance of invaders. Recently, C-type lectins have been assorted into 17 subgroups in vertebrates according to their structural and functional similarities (Zelensky and Gready, 2005), in which collectin is one subgroup serving as PRR to discriminate self and nonself (Cambi and Figdor, 2003; Medzhitov and Janeway, 2002), while selectin mainly mediates cellular adhesion to activate encapsulation and inflammation (Patel et al., 2002). For instance, mannose-binding lectin (MBL) belonging to collectin subgroup can recognize and bind terminal mannose residues and initiate the lectin pathway of complement system (Endo et al., 2006; Fujita, 2002). And P-selectin in selectin subgroup can attract and enrich leukocytes to the site of infection to quickly eliminate the pathogenic bacteria.

There are 1–4 Ca^{2+} binding sites in each CRD, among which Ca^{2+} binding site 2 has been proved to determine the binding specificity of C-type lectins in vertebrates (Weis et al., 1991, 1998; Weis and Drickamer, 1996; Zelensky and Gready, 2005). Briefly, there are two motifs existing in Ca^{2+} binding site 2. The first motif is EPN (Glu-Pro-Asn) or QPD (Gln-Pro-Asp), which is experimentally verified to determine the binding specificity of C-type lectin. The second motif is WND (Trp-Asn-Asp) which is identified to cooperate with the first motif in binding process against carbohydrates (Cambi et al., 2005; Weis and Drickamer, 1996; Weis et al., 1998). Moreover, intensive researches demonstrated that C-type lectins with EPN/WND motifs bind D-mannose or similar sugar, while C-type lectins with QPD/WND motifs bind D-galactose or similar sugar (Wang et al., 2011a; Zelensky and Gready, 2005). However, the two motifs are of great diversity of characteristics in invertebrate, and various kinds of amino acid sequence have been found in the corresponding sites of two specific motifs. For instance, EPD (Glu-Pro-Asp), QPG (Gln-Pro-Gly), QPS (Gln-Pro-Ser), YPG (Tyr-Pro-Gly), and YPT (Tyr-Pro-Thr) were found in the first motif, at the

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same time WID (Trp-Ile-Asp), WSD (Trp-Ser-Asp), WHD (Trp-His-Asp), FSD (Phe-Ser-Asp) and LSD (Leu-Ser-Asp) were found in the second motif (Wang et al., 2011a).

The number and organization of CRDs determine the affinity and spectrum of lectins to recognize and bind nonself invaders. Some vertebrate C-type lectins contained several CRDs and single CRD containing C-type lectins usually tend to form polymers to perform functions. For instance, the mannose receptor is composed of eight CRDs, whereas MBL is inclined to form polymers to activate complement system (Zelensky and Gready, 2005). To our knowledge, most reported invertebrate C-type lectins containe one single CRD (Ao et al., 2007; Belogortseva et al., 1998; Huang et al., 2015; Li et al., 2015; Mu et al., 2012). Very recently, however, several invertebrate C-type lectins with double or multiple CRDs have been proved to involve in immune responses including PAMPs and microbes binding (Yang et al., 2015), bacteria agglutination (Tian et al., 2009) and opsonization (Wang et al., 2011b). Fc-Lec2 from *Fenneropenaeus chinensis* with two tandem CRDs and CfLec-3 from *C. farreri* with three dissimilar CRDs have broad and strong affinity to recognize microbes and PAMPs (Yang et al., 2015; Zhang et al., 2009). Nevertheless, the relationship between the structure and functions of multidomain C-type lectins is still defectively understood, especially in invertebrates.

In our previous study, CfLec-4 with four CRDs was demonstrated to be involved in PAMPs recognition, microbe binding and phagocytosis enhancement against bacteria. In the current study, the four CRDs of CfLec-4 were expressed in *Escherichia coli* separately, and their PAMP binding specificities, microbe binding spectrums as well as the phagocytosis enhancement activities were examined to comprehensively explore the role of each CRD played in immune response against invaders.

2. Materials and methods

2.1. Scallop and microbes

Adults of scallop *C. farreri* with an average 55 mm of shell length were collected from a farm in Qingdao, Shandong Province, China, and maintained in the aerated seawater at 18 °C for a week before processing.

Bacteria *Micrococcus luteus*, *Staphylococcus aureus* and *Escherichia coli* was purchased from Microbial Culture Collection Center (Beijing, China). *Vibrio anguillarum* was kindly provided by Dr. Zhaolan Mo. *Yarrowia lipolytica* was kindly provided by Dr. Zhenming Chi. *Pichia pastoris* GS115 was purchased from Invitrogen.

2.2. Sequence analysis

The ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) 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 Mega4.0 program (Wang et al., 2015). Bootstrap re-sampling (1000 pseudo-replicates) was employed to test the reliability of the branching.

2.3. Preparation of recombinant proteins

The four CRDs of CfLec-4 were recombined to plasmid pET-32a and were transformed into the strains *E. coli* BL21 (DE3)-pLysS respectively. The recombinant protein rCRD1, rCRD2, rCRD3, rCRD4 and the recombinant protein Trx (designated as rTrx) were purified and quantified as described before (Huang et al., 2012).

2.4. Preparation of antibodies and western-blot analysis

The renatured protein rCfLec-4 and rTrx were immuned to 6 weeks old mice to acquire polyclonal antibody. The specificity of antibodies were detected as described previously (Huang et al., 2012).

2.5. Microbe binding assay

Gram-positive bacteria (*M. luteus,* and *S. aureus*), Gram-negative bacteria (*E. coli* and *V. anguillarum*) and fungi (*P. pastoris* and *Y. lipolytica*) were used to determine the microbe binding spectrum of rCRD1, rCRD2, rCRD3 and rCRD4 according to the method described by Lee (Lee and Söderhäll, 2001). One hundred microliters of rCRD1, rCRD2, rCRD3 and rCRD4 were incubated with microbes under rotations for 30 min at room temperature. Then the microbes were washed with TBS and finally, subjected to elution with 8% SDS. The microbe binding assay was detected by Western-blot analysis. rTrx group was employed as negative control.

2.6. The PAMPs binding assay

The PAMP binding activities of rCRD1, rCRD2, rCRD3, and rCRD4 were measured according to previous report (Huang et al., 2012). After incubated with primary antibody and second antibody, one hundred microliters of 0.1% (w/v) pnitrophenyl phosphate (pNPP) containing 0.5 mM $MgCl_2$ was added to each well for chromogenic reaction. The same concentration of rTrx was used as control. The wells filled with 100 μL of TB were used as blank. The absorbance was measured with an automatic ELISA reader at 405 nm. Each experiment was repeated in triplicates and the results were given in terms of the mean of three individual measurements ± S.E. ($N = 3$). Samples with P (sample)-B (blank)/N (negative)-B (blank) > 2.1 were considered as positive.

2.7. Phagocytosis assay

Phagocytosis assay was performed according to previous method (Huang et al., 2012). The phagocytic activity of hemocyte attached to the slide was measured using a light microscope. Two hundred hemocytes on each slide were counted, and the phagocytic rate (PR) representing the phagocytic activities was expressed as following: PR= (phagocytic hemocytes)/(total hemocytes) × 100%. For each treatment, assay was performed in three different slides for statistical analysis.

3. Results

3.1. The sequence features and phylogeny of CRD1, CRD2, CRD3 and CRD4

Multiple sequence alignment of CRDs was developed by ClustalW to identify the signature sequences of the four CRDs (Fig. 1). Each CRD contained four cysteine residues involving in the formation of the conserved internal disulfide bridges. Besides, the two N-terminus cysteine residues in all CRDs indicated that they were of long-form (Zelensky and Gready, 2005). The key motifs of Ca^{2+} binding site 2 in four CRDs were EPD/LSD, EPN/FAD, EPN/LND and EPN/YND, respectively. A phylogenetic tree was constructed by NJ method with 1000 bootstrap-test based on the multiple alignments of CRDs from bivalve lectins and other C-type lectins from *Botryllus schlosseri*, *Danio rerio*, *Gallus gallus*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Salmo salar* and *Taeniopygia guttata* (Fig. 2). Three distinct groups were separated in the phylogenetic tree. The CRDs of collectins were clustered into a branch and the CRDs of selectins

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