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A single-CRD C-type lectin from oyster *Crassostrea gigas* mediates immune recognition and pathogen elimination with a potential role in the activation of complement system

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C-type lectins (CTLs), serving as pattern recognition receptors (PRRs), are a superfamily of Ca^{2+}-dependent carbohydrate-recognition proteins that participate in nonself-recognition and pathogen elimination. In the present study, a single carbohydrate-recognition domain (CRD) CTL was identified from oyster *Crassostrea gigas* (designated as CgCLec-2). There was only one CRD within the deduced amino acid sequence of CgCLec-2 consisting of 129 amino acid residues. A conserved EPN (Glu_{246}-Pro_{247}-Asn_{248}) motif was found in Ca^{2+}-binding site 2 of CgCLec-2. The CgCLec-2 mRNA could be detected in all the examined tissues at different expression levels in oysters. The mRNA expression of CgCLec-2 in hemocytes was up-regulated significantly at 6 h post *Vibrio splendidus* challenge. The recombinant CgCLec-2 (rCgCLec-2) could bind various Pathogen-Associated Molecular Patterns (PAMPs), including lipopolysaccharide, mannan and peptidoglycan, and displayed strong binding abilities to *Vibrio anguillarum*, *V. splendidus* and *Yarrowiali polytica* and week binding ability to *Staphylococcus aureus*. It could also enhance the phagocytic activity of oyster hemocytes to *V. splendidus* and exhibited growth suppression activity against gram-positive bacteria *S. aureus* but no effect on gram-negative bacteria *V. splendidus*. Furthermore, the interaction between rCgCLec-2 and rCgMASPL-1 was confirmed by GST Pull down. The results suggested that CgCLec-2 served as not only a PRR in immune recognition but also a regulatory factor in pathogen elimination, and played a potential role in the activation of complement system.

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1. Introduction

C-type lectins (CTLs) are a superfamily of Ca^{2+}-dependent carbohydrate-recognition protein containing at least one carbohydrate-recognition domain (CRD) of about 130 amino acid residues [1–3]. They are present in almost all metazoans and serve as one major family of Pattern Recognition Receptors (PRRs) in innate immunity to play vital roles in nonself recognition and clearance of invaders by recognizing and binding to sugar ligands existing extensively on the microorganisms [1,3].

Typical vertebrate CTLs are Ca^{2+}-dependent carbohydrate binding proteins and most of them contain one CRD for ligand binding [3]. The binding of Ca^{2+} has important implications not only in maintaining CRD structure but also in ligand binding activity. Each CRD contains four Ca^{2+}-binding sites and the site 2 is considered to be involved in carbohydrate-binding specificity [3]. Based on the conserved amino acid motifs for ligand binding and calcium coordination, classical vertebrate CTLs have been further divided into two main groups, mannose-type with EPN motif in the CRD for predicted binding specificity of mannose and galactose-type with QPD motif for recognition of galactose and N-acetyl-D-galactosamine (GalNac). The CTLs without the conserved EPN or QPD motif belong to the other-types [3,4]. Vertebrate CTLs act as soluble proteins existing in tissue fluids or as cell surface receptors for microbial carbohydrates in innate immunity and play various

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functions. For example, mannose binding lectin (MBL) as a soluble protein involves in complement activation [5], while cell surface receptor Endo180 acts as recycling endocytic receptor [6].

Recently, an increasing number of CTLs have been identified in invertebrate, and most of them contain one CRD [3]. Invertebrate CTLs are diverse significantly in the amino acid sequences of ligand binding motifs. For instance, lots of novel motifs have been identified in Ca^{2+}-binding site 2 of invertebrate CRDs except for conserved ones EPN and QPD, which may contribute to the diverse function of this superfamily [7].

As important PRRs, invertebrate CTLs play important roles in recognizing and binding to PAMP and microbes, such as BmMBP from silkworm *Bombyx mori* and EsLecF from Chinese mitten crab *Eriocheir sinensis* could recognize a wide-range of microorganism, including gram positive bacteria (*Micrococcus luteus* or *Staphylococcus aureus*), gram negative bacteria (*Escherichia coli* or *Aeromonas hydrophila*) and fungi (*Saccharomyces cerevisia*e or *Pichia pastoris*) [8,9]. Some invertebrate CTLs had been reported to provide protection against pathogen invasion by mediating phagocytosis and growth suppression activity. For instance, CfLec-1 from scallop *Chlamys farreri* [10] and MCL-4 from clam *Ruditapes philippinarum* [11] could trigger the enhancement of phagocytosis. MCL-4 could also suppress the growth of *Alteromonas haloplanktis* [11], and CfLec-1 and CfLec-2 were found to restrain the growth of *M. luteus* or *E. coli* [12,13]. A short-form CTL from amphioxus could directly kill *S. aureus* and *S. cerevisiae* by binding and destabilizing the microbial membrane and subsequently leading to cell death [14]. Moreover, it had been reported that a CTL from solitary ascidian, *Halocynthia roretzi* (designated as GBL) could interact with ascidian MBL-associated serine proteases (MASPs) and further involved in the activation of complement system [15]. In comparison to the discovery of numerous CTL sequences in invertebrate, the knowledge of their function is very limited. The devotion of CRDs and motifs as well as the function of mass CTLs in the immune response of invertebrates remains concern [10,12].

The oyster *Crassostrea gigas* is one of the most important aquaculture species in the world [16]. Recently, the genome sequence of *C. gigas* has been released and more than 200 CRD containing proteins have been predicted in its genome [17]. As one of the most important group of PRRs, the functions of CTLs in *C. gigas* are still poorly understood even there is one CTL, CgCLec-1, which has been reported [16]. In the present study, another CTL with single CRD from the oyster *C. gigas* (designated as CgCLec-2) was characterized by determining its tissue localization and temporal expression in hemocytes post bacterial challenge, PAMPs recognition and bacteria binding spectrum, mediation of phagocytosis, growth suppression activity against microbes and the interaction with rCgMASPL-1 in order to further understand the structural and functional characteristics of CTLs in mollusc.

2. Materials and methods

2.1. Oysters

The adult oysters *C. gigas* with an average shell length of 13.0 cm were collected from a local farm in Qingdao, Shandong Province, China. They were cultured in the aerated seawater at 15–20 °C for 10 days before processing.

2.2. Tissue collection and immune challenge

Five tissues, including hepatopancreas, gill, mantle, hemolymph and adductor muscle were dissected from six adult oysters to investigate the mRNA distribution of CgCLec-2. The tissues were briefly washed with ice cold PBS (0.14 mol L^{-1} NaCl, 3 mmol L^{-1} KCl, 8 mmol L^{-1} Na_2HPO_4, 1.5 mmol L^{-1} KH_2PO_4, pH 7.4), transferred into 1 mL of Trizol reagent and then processed immediately.

One hundred and twenty oysters were employed in the bacterial challenge experiments. They were randomly divided into three groups with 40 individuals in each group. In the challenge and control groups, 100 μl live *Vibrio splendidus* suspension (1×10^9 CFU, in sterile sea water), or 100 μl sterile sea water was injected into the adductor muscle of oysters, respectively. The rest untreated oysters were employed as blank group. Six individuals were randomly collected from each group at 3, 6, 9, 12, 24 and 48 h after injection, respectively. The hemolymph were collected and centrifuged at 800 *g*, 4 °C for 10 min to collect the hemocytes for RNA preparation.

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from tissues using Trizol reagent according to the manufacture's protocol (Invitrogen). The first-strand synthesis was carried out based on M-MLV RT Usage information using the DNase I (Promega)-treated total RNA as template and oligo (dT)-adaptor as primer (Table 1). The reaction mixtures were incubated at 42 °C for 1 h, and terminated by heating at 95 °C for 5 min. The cDNA mix was diluted to 1:100 and stored at 80 °C for subsequent SYBR Green fluorescent quantitative realtime PCR (qRT-PCR).

2.4. cDNA cloning and sequencing of CgCLec-2 and CgMASPL-1

The primers P1 and P2 (Table 1) of CgCLec-2 and the primers P3 and P4 (Table 1) of rCgMASPL-1 were designed according to the sequence information of CgCLec-2 (JH818449) and rCgMASPL-1 (JH817732) acquired on the website of NCBI (http://www.ncbi.nlm.nih.gov/) [17]. The PCR products were cloned into pMD 19-T simple vector (TaKaRa) and confirmed by DNA sequencing. The resulting sequences were verified and subjected to cluster analysis.

2.5. Sequence, homologous and phylogenetic analysis of CgCLec-2

The cDNA sequence and deduced amino acid sequence of CgCLec-2 were analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). The protein domain was revealed by the simple modular architecture research tool (SMART) version 4.0 (http://www.smart.emblheidelberg.de/). The presumed tertiary structure was established for the CRDs using the SWISS-MODEL prediction algorithm (http://swissmodel.expasy.org/) and

Table 1
Primers used in this paper.

Primer	Sequence(5′—3′)
Oligo(dT)-adaptor	$GGCCACGCGTCGACTAGTACT_{17}$
Clone primers	
P1(forward)	ATGGATACAGAAGTGACCTCAT
P2(reverse)	CTATAAAGTTTGTTCACAGATG
P3(forward)	ATGTTCGTCAGCTTCGTAACGG
P4(reverse)	GGTCAAAGATGAAATGAAATGTTACC
RT primers	
P5 (CgCLec-2-RTF)	ATGGTCCATGTTTGAGACTTCCT
P6(CgCLec-2-RTR)	ACCTTCCGTGACATCATCTTTAC
P7 (EF-RTF)	AGTCACCAAGGCTGCACAGAAAG
P8 (EF-RTR)	TCCGACGTATTTCTTTGCGATGT
Recombination primers	
P9(forward)	CGCGGATCCATGGATACAGAAGTGACCTCAT
P10(reverse)	CCCAAGCTTCTATAAAGTTTGTTCACAGATG
P11(forward)	CGCGGATCCATGTTCGTCAGCTTCGTAACGG
P12(forward)	CCGCTCGAGGGTCAAAGATGAAATGAAATGTTACC

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