Full length article

Two novel LRR and Ig domain-containing proteins from oyster *Crassostrea gigas* function as pattern recognition receptors and induce expression of cytokines

CrossMark

Xiudan Wang [a, d], Mengqiang Wang [a], Qingsong Xu [c], Jiachao Xu [a, d], Zhao Lv [a, d], Lingling Wang [c], Linsheng Song [b, c, *]

[a] Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China
[c] Liaoning Key Laboratory of Marine Animal Immunology and Disease Control, Dalian Ocean University, Dalian 116023, China
[d] University of Chinese Academy of Sciences, Beijing 100049, China

ABSTRACT

Leucine-rich repeat (LRR) domain and immunoglobulin (Ig) domain are both competent immune recognition modules, and the immunological roles of LRR and Ig domain containing- proteins (LRRIGs) are speculated to be multifunctional and worth investigating. In the present study, two novel LRRIGs, CgLRRIG-1 and CgLRRIG-2, were identified and characterized from oyster *Crassostrea gigas*. Both of them contained an N-terminal LRR region, an Ig domain, a transmembrane region, and a C-terminal cytoplasmic tail. The mRNA transcripts of CgLRRIG-1 and CgLRRIG-2 were constitutively expressed in muscle, gill, hepatopancreas, mantle, gonad and hemocytes with the highest expression level in hepatopancreas. Their mRNA expression levels in hemocytes were significantly up-regulated after the stimulations with four PAMPs including peptidoglycan (PGN), lipopolysaccharide (LPS), glucan (GLU) and polyinosinic-polycytidylic acid (poly I:C) and one bacteria *Vibrio anguillarum*. The recombinant proteins, rCgLRRIG-1 and rCgLRRIG-2, could bind to PGN, LPS, GLU and poly I:C, and rCgLRRIG-2 exhibited higher binding affinity. Additionally, rCgLRRIG-1 and rCgLRRIG-2 could significantly induce the expression of CgTNF-1 and CgIL17-5 in cultured oyster hemocytes, and the activity of rCgLRRIG-2 was higher than that of rCgLRRIG-1. All these results indicated that CgLRRIG-1 and CgLRRIG-2 could function as immune effectors or pro-inflammatory factors as well as PRRs in oyster.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

The innate immune system provides immediate protection against infections and it is regarded as the first line of host defense in vertebrate and invertebrate [1,2]. Pattern recognition receptors (PRRs) function as the initiators of the immune response by recognizing the pathogen associated molecular patterns (PAMPs) and triggering the downstream cascade of immune reactions [1,2]. The identified PRRs of the innate immunity usually contain various domains, such as LRR domain, Ig domain, carbohydrate-recognition domains (CRD), caspase-recruitment domain and C1q domain [2–5].

The LRR domain usually consists of a chain of 2–45 LRR motifs and each LRR motif is typically 20 to 29 residues long with the consensus sequence LxxLxLxxN/CxL (x can be any amino acid, L can also be occupied by I/S/V, N can also be C/S/T and C can also be N/S) [6]. LRR domains are responsible for peptide-ligand binding and protein-protein interaction [7,8]. The proteins with LRR domains such as Toll-like receptors (TLRs), RIG-1-like receptors (RLRs) and NOD-like receptors (NLRs), play important roles in immune recognition of PAMPs from bacteria, fungi, virus, and parasite [5,9–11]. There are proteins merely containing LRR motifs (LRR-only proteins), which have been identified in some invertebrate such as amphioxus, sea urchin, shrimp and scallop [12–15]. For example, a LRR-only protein in shrimp could respond to WSSV stimulation [15]. The mRNA transcripts of LRR-only proteins in scallop were up-regulated after stimulation of four PAMPs

including PGN, LPS, GLU and poly I:C [13,14]. Ig domain containing proteins serve a variety of functions in ligand recognition, cellular adhesion, and signal transduction [16,17]. In vertebrate, proteins with Ig domain form a very large and diverse pattern recognition repertoire by V(D)J recombination [18]. In invertebrate, proteins with Ig domain exhibited various immunological functions. For example, Dscam from *Anopheles gambiae* could generate a broad range of PRRs in immune defense [19]. Ig domain containing proteins in *Crassostrea gigas,* CgSiglec-1 and CgJAM-A-L, could bind to multiple PAMPs and various microorganisms to regulate phagocytosis, apoptosis and cytokine release [20,21]. A typical LRRIG consists of an N-terminal LRR region, one or more central Ig domains, a transmembrane region, and a C-terminal cytoplasmic tail [12]. In vertebrate, 30 LRRIG proteins were identified and most of them functioned in the nervous system [12]. In invertebrate, 20 LRRIG models have been identified in sea urchin genome and 240 LRRIG models have been identified in amphioxus genome, but their functions are still far from well understood [12]. On account of the importance of the LRR domain and Ig domain in immune response, the biological activities and the immunological functions of LRRIGs are worth investigating.

Recently, the immune system of Pacific oyster, *C. gigas* has been widely explored not only for its economic and evolutionary importance but also for its ability to handle complex environmental changes in estuaries and intertidal zones [2]. Especially, the recently released genome of *C. gigas* provides valuable information for the study on the immune system of Pacific oyster [22]. In the present study, two LRRIGs were identified in *C. gigas,* (defined as CgLRRIG-1 and CgLRRIG-2, respectively) with the main objectives, (1) to examine their expression profiles in different tissues and their alternations after PAMPs stimulation, (2) to validate their binding activities towards PAMPs and their modulation of cytokine expression, and (3) to compare their structural features and explore the diversification of LRRIGs in invertebrate.

2. Materials and methods

2.1. Animals and pretreatment

Oysters *C. gigas* with an average shell length of 13.0 cm were collected from a local farm in Qingdao, China. They were pre-punched by the side of shell and then maintained at 20 °C in the aerated seawater for one week.

2.2. Immune stimulation and sample collection

Approximately 300 oysters were employed and randomly divided into 6 groups for PAMPs and bacteria stimulations. Four different PAMPs and one Gram negative bacteria *Vibrio anguillarum* were used for immune stimulation. The oysters in each group (50 oysters each group) received an injection of 100 μL PGN (77140, Fluck, USA), LPS (L2630, Sigma Aldrich, USA), GLU (G5011, Sigma Aldrich, USA) or poly I:C (P1530, Sigma Aldrich, USA) at the concentration of 0.5 mg mL^{-1} in phosphate buffered saline (PBS, pH 7.4) respectively. Oysters in the negative control group received an injection of PBS. The bacteria were cultured in 2216E media at 16 °C for 24 h and then harvested by centrifugation at 4000g, 4 °C for 10 min. The pellets were washed with PBS for three times and re-suspended in PBS to a final concentration of about 1×10^8 CFU mL^{-1}. Then 50 oysters in the bacteria stimulation group received an injection of 100 μL bacteria. The oysters were returned to seawater tanks after air exposure for 10−15 min. The hemolymphs from oysters were collected at 3, 6, 9, 12, 24 and 48 h post injection and centrifuged at 800 g, 4 °C for 10 min to harvest the hemocytes. Muscle, gill, hepatopancreas, mantle, female gonad, male gonad and hemocytes were collected to determine mRNA expression levels of CgLRRIG-1 and CfLRRIG-2 in different tissues. Tissues from two oysters were pooled together as one sample and there were three samples for each determination.

2.3. RNA isolation and cDNA synthesis

The total RNA isolation and cDNA synthesis were performed according to previous study [23]. The obtained cDNA mix was diluted 40-fold and stored at −80 °C for subsequent quantitative real-time PCR (qRT-PCR).

2.4. cDNA cloning of CgLRRIG-1 and CgLRRIG-2

The cDNA sequences of CgLRRIG-1 (EKC19646.1) and CgLRRIG-2 (EKC19645.1) were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Two pairs of primers, CgLRRIG-1-F and CgLRRIG-1-R, CgLRRIG-2-F and CgLRRIG-2-R were designed to clone the cDNA sequences. The PCR products were inserted into pMD 19-T simple vector (TaKaRa), transformed into *Escherichia coli* trans5α (TransGen) and confirmed by DNA sequencing with pMD19-T simple vector primers M13-47 and RV-M.

2.5. Sequence analysis and 3D-model structure prediction

The cDNA sequences and deduced amino acid sequences of CgLRRIG-1 and CgLRRIG-2 were analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). The signal peptide was predicted via SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP). LRR motifs were predicted by LRRFinder 2.0 (http://www.lrrfinder.com). Ig domains were determined using the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/). The 3D-model structure prediction was performed on Zhang lab (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). BLAST and PSI-BLAST algorithms were used to search for sequences related to CgLRRIG-1 and CgLRRIG-2. The following nine genes including leucine rich repeat containing 24 (LRRC24) *Mus musculus* (accession no. AAI16887.1), LRRC 24 *Homo sapiens* (accession no. BAD97811.1), LRRC24 *Danio rerio* (accession no. XP_694151.2), LRRC24-like *Takifugu rubripes* (accession no. XP_003973845.1), LRRC24 *Monomorium pharaonic* (accession no. XP_012524524.1), leucine-rich repeat and immunoglobulin-like domain-containing protein 1 (LRRIG-1) *Strongylocentrotus purpuratus* (accession no. XP_011671814.1), leucine-rich repeat neuronal protein 1-like (LRR1 like) *Takifugu rubripes* (accession no. XP_003973733.1), leucine-rich repeat and fibronectin type III domain-containing protein 1-like protein (LRRC1-like) *Aplysia californica* (accession no. XP_012936245.1), LRRC24-like *Aplysia californica* (accession no. XP_005095344.1) were selected for phylogenetic analysis by Neighbor-Joining methods with Poisson model in Mega 5.0 based on the full lengths of CgLRRIG-1 and CgLRRIG-2. The reliability of the branching was tested using bootstrap of 1000.

2.6. Quantitative real time PCR analysis (qRT-PCR) of the mRNA expression level

The relative mRNA expression levels of CgLRRIG-1 and CgLRRIG-2 were detected by qRT-PCR with SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara, RR420A). Two pairs of specific primers, CgLRRIG-1-qRT-F and CgLRRIG-1-qRT-R, CgLRRIG-2-qRT-F and CgLRRIG-2-qRT-R (Table 1) were used to amplify fragments of 121 bp and 95 bp, respectively. The *C. gigas* elongation factor (CgEF) fragment amplified with primers CgEF-qRT-F and CgEF-qRT-R (Table 1) was