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LRFN (leucine-rich repeat and fibronectin type-III domain-containing protein) recognizes bacteria and promotes hemocytic phagocytosis in the Pacific oyster *Crassostrea gigas*

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ABSTRACT

In bivalve mollusks, circulating hemocyte mediated phagocytosis is one of the primary ways to eliminate invading microbes. Here, we have identified one CgLRFN (leucine-rich repeat and fibronectin type-III domain-containing protein) in the *Crassostrea gigas* as a novel transmembrane LRR (Leucine-rich repeat) domain containing protein in *C. gigas*, homologous to the jawless fish VLR protein, that plays an important role in recognizing bacteria and promoting hemocytic phagocytosis. Tissue distribution analysis of *Cg*LRFN in Pacific oyster showed that it is widely expressed in various tissues like the gills, adductor muscles, digestive glands, gonads, heart and in the hemocytes. Furthermore, infection of Pacific oysters with two marine *Vibrio* strains *V. alginolyticus* and *V. parahaemolyticus* was found to significantly increase *Cg*LRFN expression in the hemocytes. Analysis of subcellular localization showed that *Cg*LRFN is primarily localized in the cell membrane. Additionally, *Cg*LRFN was found to be able to bind both the bacterial strains, indicating its possible role as a cell surface receptor. Flow cytometry analysis revealed that *Cg*LRFN coated bacteria was phagocytosed by oyster hemocytes at a significantly higher rate compared to the uncoated bacteria. Finally, RNAi mediated knockdown of *Cg*LRFN *in vivo* resulted in reduced clearance of both the bacterial strains from the oyster hemolymph. Overall, our study demonstrates that *Cg*LRFN acts as a pattern recognition receptor for *Vibrio spp.* and promotes hemocytic phagocytosis in the Pacific oyster, which is critical for understanding the mechanism of bacterial infection in lower invertebrates, and also contributes to disease management of this economically and ecologically important marine mollusk.

1. Introduction

Pacific oysters (*Crassostrea gigas*) are one of the most widely popular edible aquaculture species, accounting to a huge economic market all over the world. These marine bivalves are also highly ecologically important since oysters possess an open circulatory system and have the capacity to filter water, thereby clearing particulates and pollutants [1,2]. However, this filter-feeding system in turn exposes the mollusks to various invasive and pathogenic microbes including bacteria and viruses [3]. These microbes, especially *Vibrio* pathogens such as *V. parahaemolyticus* and *V. alginolyticus*, mainly inhabit coastal areas and pose a serious threat to human health as well as to the oysters [4,5]. Majority of food-borne diseases causing severe gastroenterotitis in humans occur due to consumption of raw seafood which carry these pathogens [6]. Hence understanding the underlying mechanism of the invertebrate host defense mechanisms in these mollusks is critical for efficient management and prevention of food-borne diseases in humans.

Hemocytes play a critical role in invertebrate cellular immunity by their ability to phagocytose, encapsulate and kill the microbes [7]. Bivalve hemocytes are structurally and functionally similar to the vertebrate macrophage/monocyte lineage, and have been shown to produce several reactive oxygen intermediates, nitric oxide and other lysosomal

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enzymes for destruction of phagocytosed particles [8,9]. As the first step for phagocytosis, the recognition of pathogens is necessary, and is generally mediated through recognition of highly repeated conserved microbial structures termed pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRR) in the host [10,11]. TLRs (Toll-like Receptor) are a group of type I membrane receptors which act as PRR and are involved in both vertebrate and invertebrate immunity through recognition of these PAMPs [12,13]. Binding of TLR to the PAMPs is known to occur through specialized Leucine-rich repeat domains (LRR), which are found to be evolutionarily conserved in many proteins involved with plant, invertebrate and mammalian immunity [14,15]. Similarly, the Leucine rich and fibronectin type III domain containing proteins (LRFN) are another class of type I transmembrane glycoproteins consisting of LRR domains, an immunoglobulin C2-like (IgC2) domain, a fibronectin type III (FNIII) domain, a TM domain, and a cytoplasmic C-terminal tail which play essential roles in neural outgrowth, monocyte migration and synapse formation in mammals [16,17]. However, it is still unknown whether any LRFN homologue is involved in innate immunity in invertebrates. Interestingly, the LRFN family of proteins were found to have strong structural resemblance to the VLR (Variable Lymphocyte Receptor) protein in jawless fish. The jawless-fish VLR also consists of an N-terminal SP, several LRR domains along with both N-terminal and C-terminal LRR domains with a hydrophobic tail (HT) [18]. Previous evidence showed that VLR gene with highly variable LRR cassettes brings out the diversity necessary for recognition of foreign antigens, thereby playing a functional role in adaptive immunity [19]. Owing to the presence of the LRR domains and the strong structural homology between LRFN and VLRs, we hypothesized that LRFN might be functionally involved in immune defense against pathogenic microbes.

In current study, we demonstrated one LRFN gene in the pacific oyster *Crassostrea gigas*, which shares high similarity of domain architecture with vertebrate's homologues and jawless-fish VLR. To explore the possible role of oyster LRFN genes in innate immunity, we investigated its ability in bacterial binding and hemocytic phagocytosis. Given the economic importance of this bivalve mollusk, our findings have immense significance in understanding the immune defense mechanism of oysters against bacterial infection and can be utilized for control and prevention of oyster diseases during the aquaculture.

2. Methods

2.1. Cloning, sequencing and bioinformatic analysis of full-length CgLRFN

The ORF sequence of *Cg*LRFN were identified from genome database of *C. gigas* [20] On the basis of this sequence, the GeneRacer™ kit (Invitrogen, CA, USA) was used to obtain its 3′and 5′ends according to manufacturer's protocol. The ORF of *Cg*LRFN was amplified using primer pairs LRFN-F5 and LRFN-R5 (Table 1) harboring the restriction sites, *EcoR* I and *Xho* I, respectively. The PCR products was cloned into pGEM-T Easy Vector (Promega, WI, USA) and sequenced by ABI Prism 3730 DNA sequencer (Perkine Elmer, Wellesley, MA, USA). The sequence of *Cg*LRFN homologues across species were analyzed using the BLAST algorithm at NCBI(http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). The protein domains were predicted by SMART (http://smart.embl-heidelberg.de).

2.2. Oysters, tissue collection and bacterial challenge

The Pacific oysters (two years old with average shell length of 90–110 mm) were purchased from Qingdao fishery market (Shangdong province, China), which were acclimatized at 22–25 °C in tanks with circulating seawater two weeks prior to experiments. The oysters were fed twice with mixture of Tetraselmis suecica and Isochrysis galbana 1:1 for each day. For the tissue distribution analysis, different tissues were extracted from gills, mantle, adductor muscle, digestive gland, gonads and hemocytes from three healthy oysters, separately. For the bacterial challenge analysis, each oyster was challenged by injecting 100 μl of *Vibrio alginolyticus (ATCC 17749T)* or *Vibrio parahaemolyticus 5HP* (10^7 × CFU) into adductor muscles, and the control groups were injected with equal volume of PBS. Hemocytes were collected from the pericardial cavity through the adductor muscle at 3, 6, 12, 24, 48, 72 h after challenge, and then immediately centrifuged 1000*g* for 5 min at 4 °C to separate the hemocytes cells from plasma. Five individuals were randomly sampled in each group at every time point.

2.3. Isolation of total RNA and real-time quantitative RT-PCR

Total RNAs were isolated from multiple tissue or hemocytes with TRIzol Reagent (Invitrogen, USA) and the integrity was checked by agarose gel electrophoresis. To synthesize cDNA, 1 μg of total RNA was subjected to reverse transcription using PrimeScript™ RT Reagent Kit (TaKaRa, Japan). The *CgLRFN* expression profile was measured using Quantitative real-time PCR. The reference gene GAPDH was employed here to normalize the expression level. All primers for real-time PCR analysis are listed in Table 1. The qPCR was performed in the Light-Cycler 480 (Roche) platform with 20 μL reaction system, containing 10 μL of 2 × Master Mix (Roche, USA), 0.4 μL of each of primer (10 mM), 1 μL of 1:10 diluted cDNA, and 8.2 μL of water. The dissociation curve was analyzed to confirm specificity of amplicons. Each sample was carried out in triplicates.

2.4. Cell culture and transfection

The plasmids expressing full length *Cg*LRFN were transfected into HEK293T cell for subcellular localization analysis. The endotoxin-free plasmids were obtained using Ezgene EndoFree Plasmid Kit (BioMIGA, USA). The HEK293T cell were cultured in high-glucose DMEM (Gibco) containing10% FBS and 10^5 U/L penicillin and 100 mg/L streptomycin at 37 °C in a humidified incubator under 5% CO2 as described previously [21]. The transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For subcellular localization, HEK 293T cells were plated on cover slips in 6-well plates and transfected with 2 μg of CgLRFN-GFP plasmid. 48 h post-transfection, HEK 293T cells were washed with PBS twice, then fixed with 4% paraformaldehyde for 10 min. Nuclei were stained with 4, 6-diamino-2-phenylindole (DAPI, 1 mg/ml). Coverslips were then mounted onto slides with 0.64 g/ml 1, 4-diazabicyclo-octane and 22% glycerol in PBS, and visualized through a fluorescent microscope (Leica, Germany).

2.5. Plasmid construction, expression and purification of recombinant CgLRFN

For downstream experiments, we decided to utilize the extracellular part of *Cg*LRFN (ep-*Cg*LRFN). The ep-*Cg*LRFN sequence was amplified using primersLRFN-F4 and LRFN-R4 with the restriction sites, *EcoR* I and *Xho* I. The target amplicon was digested and inserted into the prokaryotic expression vector pGEX-4T-1. Subsequently, the resulting clones were confirmed by DNA sequencing, and then transformed into *E. coli* BL21 (DE3). Positive clones were incubated in LB medium with 100 mg/ml ampicillin at 37 °C with shaking at 200 rpm. When the OD600 of culture medium reached0.5–0.6, IPTG (1.0 mM) was added into the medium for 4 h at 16 °C in order to induce the expression of recombinant protein. The empty vector without any insert was used as negative control. The recombinant ep-*Cg*LRFN protein were purified using Glutathione Sepharose 4B (GE Healthcare, USA) and analyzed using 12% sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).