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Molecular characterization and functional analysis of a novel C-type lectin receptor-like gene from a teleost fish, *Plecoglossus altivelis*

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ABSTRACT

C-type lectin-like receptors (CLRs) are important pathogen pattern recognition molecules that recognize carbohydrate structures. However, the functions of these receptors in fish keep less known. In this study, we characterized a novel CLR from a teleost fish, *Plecoglossus altivelis* (ayu), tentatively named PaCD209L. The cDNA of PaCD209L is 1464 nucleotides (nts) in length, encoding a polypeptide of 281 amino acid residues with a calculated molecular weight of 31.5 kDa. Multiple alignment of the deduced amino acid sequences of PaCD209L and other related fish CLRs revealed that the PaCD209L sequence had typical characteristics of fish CLRs, but without Ca^{2+}-binding sites. Sequence comparison and phylogenetic tree analysis showed that PaCD209L shared the highest amino acid identity (44%) with rainbow trout (*Oncorhynchus mykiss*) CD209 aE PaCD209L transcripts were detected in all of the tissues examined, mainly expressed in the brain and heart. Upon *Vibrio anguillarum* infection, PaCD209L transcripts were upregulated in all tested tissues and in monocytes/macrophages (MO/MΦ). We prepared recombinant PaCD209L (rPaCD209L) by prokaryotic expression and raised antiserum against PaCD209L. Western blot analysis revealed that native PaCD209L was glycosylated, and its protein expression significantly increased in ayu MO/MΦ upon *V. anguillarum* infection. In addition, rPaCD209L was able to bind Gram-positive and Gram-negative bacteria in the absence of Ca^{2+}. After PaCD209L was blocked by anti-PaCD209L IgG, the phagocytosis and bacterial killing activity of MO/MΦ significantly decreased. These results suggest that PaCD209L plays an important role in the regulation of MO/MΦ functions in ayu.

1. Introduction

Microorganisms that invade a vertebrate host are initially recognized by phagocytic cells (e.g., monocytes/macrophages [MO/MΦ], neutrophils, dendritic cells) through their pattern-recognition receptors (PRRs), leading to activation of the innate immune response [1]. Each PRR can recognize a group of microbial components with a similar structural pattern, and thus, a limited number of PRRs are sufficient for the surveillance of almost all microbial pathogens [2]. As a major class of PRRs, C-type lectin receptors (CLRs) whose key importance in the immune system has recently been recognized [3,4]. CLRs comprise a large family of receptors that bind to carbohydrates in a calcium-dependent manner. These proteins generally contain at least one carbohydrate recognition domain (CRD), which forms a characteristic double-loop structure, disulfide-bond positions, and calcium-binding sites. However, some CRDs reportedly bind to carbohydrates in a calcium-independent manner [3].

To date, many fish CLR genes have been sequenced, such as a mannose receptor (MR) gene in seabream (*Sparus aurata* L.) [5] and grass carp (*Ctenopharyngodon idella*) [6], natural killer cell CLRs in *Paralabidochromis chilotes* and Nile tilapia (*Oreochromis niloticus*) [7], CLR-like protein A, B, and C genes in Atlantic salmon (*Salmo salar*) [8], immune-related, lectin-like receptor genes gene in zebrafish (*Danio rerio*) [9,10], and a CLR-like protein gene in ayu (*Plecoglossus altivelis*) [11]. Fish CLRs have been reported to be involved in pathogen-associated molecular patterns (PAMPs) recognition, phagocytosis and clearance of bacteria by phagocytic cells [5–11]. Although the functions of CLRs have been extensively studied in mammals, the role of fish CLRs in regulation of the immune system keeps less known.

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Ayu (sweetfish), the sole member of the Osmeriformes family Plecoglossidae, is edible and widely cultured in East Asia. In recent years, the rapid development of ayu culture in China has suffered from vibriosis caused by *Vibrio anguillarum* [12,13]; the disease outbreak has resulted in mass mortality of ayu. The innate immune system is a fundamental defense mechanism of fish and the first line of defense against a broad spectrum of pathogens [14]. Therefore, it is important to study the mechanisms underlying the regulation of the immune system of fish and its response against pathogens. Because CLRs play important roles in recognizing foreign pathogens and transducing signals, we focused our studies on the characterization and functional analysis of this receptor in ayu. To this end, we determined the cDNA sequence of a novel CLR gene from ayu. Recombinant PaCD209L (rPaCD209L) was prokaryotically expressed and used for raising antiserum. The relationship between PaCD209L gene expression and *V. anguillarum* infection was investigated. In addition, the pathogen recognition activity of PaCD209L and its effect on phagocytosis and intracellular bacterial killing of MO/MΦ were also determined.

2. Materials and methods

2.1. Fish rearing

Healthy ayu, weighing 40–50 g each, were purchased from a commercial farm in Ninghai county, Ningbo city, China. Fish were kept in 100 L tanks at 20–22 °C, and were acclimatized to laboratory conditions for 2 weeks before experiments. All of the experiments were approved by the Committee on Animal Care and Use and the Committee on the Ethics of Animal Experiments of Ningbo University.

2.2. Primary culture of ayu head kidney-derived MO/MΦ

Ayu head kidney-derived MO/MΦ were isolated as previously described [15]. Briefly, fish were sacrificed by anesthetic overdose (50 mg/ml; 0.03% [v/v] ethylene glycol monophenyl ether). The head kidney-derived MO/MΦ were aseptically extracted, collected, and meshed in RPMI 1640 medium (Invitrogen, Shanghai, China) supplemented with 2% fetal bovine serum (FBS; Invitrogen), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), and heparin (20 U ml^{-1}). The head-kidney leukocyte-enriched fractions were obtained using a Ficoll (Invitrogen) density gradient. The leukocyte fractions were collected and incubated overnight at 24 °C. After washing off non-adherent cells, the attached cells were incubated with RPMI 1640 medium containing 6% FBS, 4% ayu serum, and 1% penicillin/streptomycin. According to the morphological characteristics observed after Giemsa staining, more than 96% of adherent cells were MO/MΦ.

2.3. Sequence analysis

The cDNA sequence of PaCD209L was obtained from previous MO/MΦ transcriptome sequencing [16]. The authenticity of the PaCD209L cDNA sequence was confirmed by PCR amplification, cloning, and additional sequencing using an ABI 3730 automated sequencer (Invitrogen). The similarity of the obtained sequence to other known ones was analyzed by homology searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The cleavage site of signal peptides was predicted using the SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP/). The transmembrane helices were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The potential N-glycosylation sites were predicted using NetNGlyc1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). The potential disulfide bonds and their positions were predicted using the Scanprosite program (http://www.expasy.ch/tools/scanprosite/). Multiple sequence alignment was analyzed using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/), and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.0 [17].

2.4. Bacterial challenge

V. anguillarum challenge was carried out as previously reported [18]. Briefly, bacteria were grown in nutrient broth on a rotary shaker at 28 °C, and were harvested in the logarithmic phase of growth, which was monitored by the optical density assay. The cells of *V. anguillarum* were washed, resuspended, and diluted to the appropriate concentration in sterile PBS. Fish were challenged by intraperitoneal injection with 1.2×10^4 colony forming units (CFUs) *V. anguillarum* (in 100 μl PBS) per fish, and PBS alone was used as a control. At 0, 4, 8, 12, and 24 h post-injection, ayu tissues, including the brain, gill, heart, head kidney, liver, spleen, skin, and intestine, were collected and preserved at −80 °C until subsequent use.

2.5. Real-time quantitative PCR assay

Real-time quantitative PCR (qPCR) assay was performed in an RT-Cycler™ Realtime Fluorescence Quantitative PCR thermocycler (CapitalBio, Beijing, China). Total RNA was extracted from ayu tissues and MO/MΦ using RNAiso reagents (TaKaRa, Dalian, China), following by deoxyribonuclease I digestion to eliminate genomic DNA. The specific primers for PaCD209L cDNA detection were: PaCD209L-T(+) 5′- GGTCTCCATCTCAGGGAACA -3′ and PaCD209L-T(−) 5′- GGCTGCAGTCTT GAAAGGTC -3'. Primers pActin-F 5′-TCGTGCGTGACATCAAGGAG-3′ and pActin-R 5′-CGCACTTCATGATGCTGTTG-3' [18] were used to amplify the cDNA of a housekeeping β-actin gene, which was used as an internal control. Amplifications were performed in triplicate for each sample using the SYBR® Premix Ex Taq™ (Perfect Real-Time) kit (TaKaRa). The reaction mixture was incubated for 300 s at 95 °C, followed by 40 amplification cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The mRNA expression of PaCD209L was normalized against that of β-actin using the $2^{-\Delta\Delta CT}$ method [19].

2.6. Prokaryotic expression of PaCD209L and antiserum preparation

The sequence encoding the extracellular domain of PaCD209L was amplified from an MO/MΦ cDNA template with the following primers: PaCD209L-P(+) 5′- CGGATCCATGGAGATGAAAGACATAA -3′ and PaCD209L-P(−) 5′- GGAATTCCTAGGCCGGGGCTTCTA -3' (underlined bases are *BamH* I and *EcoR* I sites, respectively). After restriction enzyme digestion, the amplicon was orientedly inserted into the pET32a vector. The recombinant pET32a-PaCD209L plasmid was then transformed into *Escherichia coli* BL21(DE3) pLysS, and its N-terminal 6 × His-tag (rPaCD209L) expression was induced with IPTG. rPaCD209L was purified using a nickel-nitrilotriacetic acid (Ni-NTA) column (QIAGEN, Hilden, Germany), and the purified protein was used to immunize BALB/c mice to produce antiserum. Protein A agarose beads (Invitrogen) were used to precipitate IgG from the antiserum as previously described [20]. A healthy mouse IgG (isotype IgG) was used as an isotype control. The purity of IgG was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

2.7. Western blot analysis

Protein samples from MO/MΦ in respond to bacterial infection were analyzed by Western blot analysis as previously reported [15].