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Short communication

Genomic characterization and transcriptional evidence for the involvement of complement component 7 in immune response of rock bream (*Oplegnathus fasciatus*)

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ABSTRACT

The complement component 7 (C7) is the central mediator of pathogenic attack at the membrane surface and its binding to the C5b-7 complex triggers cytolytic signaling. In this study, C7 of rock bream (*Oplegnathus fasciatus*) was identified (*Rb-C7*) and characterized at the genomic level. The *Rb-C7* gene contains 18 exons and 17 introns and is composed of a 2490 bp complete open reading frame (ORF). The encoded polypeptide (830 amino acids) contains a number of well-conserved C7 signature domains. Important putative transcription factor binding sites, including those for NF-κB, SP-1, C/EBP, AP-1 and OCT-1, are present in the 5′-flanking region of *Rb-C7*. Phylogenetic analysis revealed a close proximity of *Rb-C7* with the orthologues in tilapia and Japanese flounder. Quantitative real-time PCR (qPCR) analysis confirmed constitutive *Rb-C7* expression throughout all the examined tissue of healthy rock bream, with highest expression in liver. In immune challenge experiment, *Rb-C7* expression was up-regulated in head kidney and liver in response to *Edwardsiella tarda*, *Streptococcus iniae*, lipopolysaccharide and rock bream iridovirus (RBIV). Furthermore, significant increases of both intracellular expression level and the number of *Rb-C7*-expressing cells were detected by *in situ* hybridization assay in head kidney and liver tissues upon *E. tarda* infection. These results suggested that *Rb-C7* is lytic pathway gene in complement system and its transcriptional regulation may be an important immune response in pathogenic defense mechanism of rock bream.

1. Introduction

The complement system is an integral part of the host immune system and plays an immunoregulatory role at the interface of innate and acquired immune responses (Morgan et al., 2005). Complement activation via the classical, lectin or alternative pathways leads to formation of the membrane attack complex (MAC) on the surface of complement-opsonized cells (Nakao et al., 2011), ultimately resulting in lysis of the targeted foreign cell. MAC itself is a glycoprotein aggregate, composed of the terminal complement component (TCC) plasma proteins C5b, C6, C7, C8, and C9 that regulate the lytic pathway of the complement system (Muller-Eberhard, 1986). The genes of the C6–C9 TCCs and perforins share the unique membrane attack complex/perforin-like (MACPF) domain; moreover, their proteins exhibit high degrees of sequence similarity and all possess thrombospondin (TS), low-density lipoprotein receptor (LDLR), and epidermal growth factor precursor (EGFP) structural motifs (DiScipio et al., 1988; Hobart et al., 1995). Despite of the remarkable similarity in secondary structure, the TCCs differ in size and complexity (Li et al., 2007).

Activation of the lytic pathway occurs when C5b associates with the target cell's membrane and undergoes conformational changes

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that expose its C6 binding site. The C5b-6 complex is then capable of binding to C7. Upon C7 binding to the C5b-6 dimer, the membrane binding sites are made available, which helps to stably incorporate the complex into the target membrane (Wurzner, 2000). Formation of the C5b-7 complex (known as the intermediate complex) can facilitate further interactions with other proximal cells, initiating the process of reactive lysis (Thompson and Lachmann, 1970; Wurzner, 2000). Availability of C7 acts as the rate-limiting step of the membrane attack process, since unbound C5b-6 dissociates from the membrane without activating the cytolytic signal (Gonzalez et al., 2003). When the C5b-7 complex binds to C8 and C9 to form the complete C5b-9 complex, phosphorylation of the Bcl-2-associated death promoter (BAD) protein is induced, which leads to blockade of the FLICE-inhibitory protein (FLIP) and allows for subsequent activation of caspase-8 (Corallini et al., 2009).

To date, several genes in lytic pathway have been characterized at the molecular level, both in mammalian species and animals that serve as mammalian food sources, such as fish. In the teleost fishes, C7, C8β and C9 have been defined in trout (Kazantzi et al., 2003; Papanastasiou and Zarkadis, 2005; Tomlinson et al., 1993; Zarkadis et al., 2005), C6 (Shen et al., 2011) and C7 (Shen et al., 2012) in grass carp, C8β and C9 in Japanese flounder (Katagiri et al., 1999), C8β in carp (Nakao et al., 1996), and C9 in puffer fish (Yeo et al., 1997). In addition, C7 and C9, along with their promoter regions, have been predicted in grass carp via bioinformatics analysis (Li et al., 2007; Shen et al., 2012). However, no comprehensive immune-challenge study had been conducted on any of these complement factors for any of these fishes.

Rock bream (*Oplegnathus fasciatus*) is one of the most economically important marine fish species of South Korea. In recent years, however, rock bream mariculture in Korea has experienced considerable economic losses due to an alarming increase in prevalence and virulence of pathogenic infections (Zenke and Kim, 2008). Gaining a detailed understanding of the rock bream immune system is critical to develop effective disease control strategies. In this study, the genomic structure of *Rb-C7* and its evolutionary relationship were determined. Furthermore, tissue-specific expression analysis was carried out to determine the constitutive expression profile and immune responsiveness of *Rb-C7* to bacteria (Gram-positive and -negative), lipopolysaccharide (LPS) endotoxin, and virus by qPCR. Finally, *Rb-C7* mRNA level and *Rb-C7* expressing cell populations of healthy and *Edwardsiella tarda* infected fish were investigated in head kidney and liver tissue by *in situ* hybridization.

2. Materials and methods

2.1. Isolation of the Rb-C7 cDNA clone and identification of genomic sequence

A cDNA sequence with the highest homology to known complement component 7 was identified by a BLAST search of the NCBI database from previously established cDNA library (Altschul et al., 1990). Thereafter, previously constructed BAC genomic library of rock bream was screened by a PCR-based method with sequence-specific primer pairs (forward: TCAGCTTGGAGCTCTGCG ATGTAT and reverse: AGATTGTCCCTGGTTTGCAGTCCT). A positive clone was isolated and purified with the QIAGEN plasmid midi kit (Germany) and confirmed by pyro-sequencing (454 GS-FLX). The *Rb-C7* genomic structure was determined by cDNA alignment using the Spidey mRNA-to-genomic alignment program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/).

2.2. Sequence characterization

The orthologue sequences of *Rb-C7* were obtained by BLAST-P of the NCBI and Ensembl databases (Altschul et al., 1990). Prediction of domains and motifs of *Rb-C7* protein was carried out using the PROSITE profile database (Bairoch et al., 1997) and SMART protein database (Letunic et al., 2009). Identity, similarity, and gap percentages were calculated by the EMBOSS pairwise alignment algorithms (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Signal peptide sequence was identified using the SignalP v3.0 server (Bendtsen et al., 2004). Promoter analysis was carried out with the bioinformatic programs of TFSearch v1.3 (Heinemeyer et al., 1998), Alibaba v2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html), and Neural Network Promoter Prediction in Berkeley Drosophila Genome Project (BDGP) (Hoskins et al., 2011). Multiple sequence alignment was performed with ClustalW v2.0 using known amino acid sequences of C7 from other organisms (Thompson et al., 1994). The phylogenetic tree was constructed with the molecular evolutionary genetic analysis (MEGA) software package v5.0 using the neighbor-joining (NJ) method (Tamura et al., 2011) and bootstrapping values taken from 5000 replicates.

2.3. Experimental fish, immune challenge and collection of tissues

Healthy rock breams (~50 g) were obtained from the Ocean and Fisheries Research Institute (Republic of Korea) and acclimatized to laboratory conditions in 400 L tanks for one week prior to any experimentation. For the expression analysis, three healthy fish were dissected for collection of tissues from gills, liver, heart, spleen, intestine, head kidney, kidney, skin, muscle, and brain. The isolated tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C. Blood samples (0.75–1.0 mL/fish) were collected from the caudal vein (at 2.5 cm from the caudal fin) and immediately centrifuged at 3000*g* (4 °C) for 10 min to harvest the peripheral blood cells.

To determine the immune responses of *Rb-C7* gene transcription, pathogenic bacteria (*E. tarda* and *Streptococcus iniae*), purified LPS (055:B5 from *Escherichia coli*; Sigma), and live RBIV were used as immune-stimulants in time-course experiments as described in previous studies (Wan et al., 2012). Rock bream head kidney and liver tissue samples were collected from three randomly selected fish for each of the immune-challenged groups and PBS-injected controls at 3, 6, 12, 24, and 48 h post-challenge.

2.4. RNA extraction, cDNA synthesis, qPCR analysis

Total RNA extraction and cDNA synthesis were carried out from the respective tissues as described previously (Whang et al., 2011). The synthesized cDNA was applied as template to the qPCR with gene-specific primers to amplify *Rb-C7* (forward: TCAGCTTGGAGC TCTGCGATGTAT and reverse: AGATTGTCCCTGGTTTGCAGTCCT) and the β-actin reference gene (forward: TCATCACCATCGGCAAT-GAGAGGT and reverse: TGATGCTGTTGTAGGTGGTCTCGT). Triplicate reactions were performed for each cDNA sample. A reaction carried out without cDNA sample served as the negative control. Finally, the C_t values were converted into relative expression levels by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The expression level for each of the tissues examined was normalized to that of the rock bream β-actin gene. For the tissue distribution analysis in healthy rock bream, the relative mRNA levels were then compared with muscle expression levels. For the expression profiles in tissues from immune-challenged fish, the β-actin normalized mRNA levels were compared with the levels detected in the respective PBS-injected controls to calculate the fold-difference for each tissue. All data are expressed as mean ± SD and significant differences of each time group vs. control (0 h) were analyzed by Student's *t*-test with the SPSS statistical software package (v16.0; SPSS Inc., USA). A *P*-value <0.05 was considered statistically significant.