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Molecular cloning, characterization of one key molecule of teleost innate immunity from orange-spotted grouper (*Epinephelus coioides*): Serum amyloid A

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ABSTRACT

The orange-spotted grouper (*Epinephelus coioides*), a favorite marine food fish, is widely cultured in China and Southeast Asian countries. However, little is known about its acute phase response (APR) caused by viral diseases. Serum amyloid A (SAA) is a major acute phase protein (APP). In this study, a new SAA homologous (EcSAA) gene was cloned from grouper, *E. coioides*, by rapid amplification of cDNA ends (RACE) PCR. The full-length cDNA sequence of SAA was 508 bp and contained a 363 bp open reading frame (ORF) coding for a protein of 121 aa. Similar to other fish known SAA genes, the EcSAA gene contained four exons and three introns. Quantitative real-time PCR analysis revealed that EcSAA mRNA is predominately expressed in liver and gill of grouper. Furthermore, the expression of EcSAA was differentially up-regulated in liver after infection with *Staphyloccocus aureus*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Saccharomyces cerevisiae* and Singapore grouper iridovirus (SGIV). Recombinant EcSAA (rEcSAA) was expressed in *Escherichia* BL21 (DE3) and purified for mouse anti-EcSAA serum preparation. The rEcSAA fusion protein was demonstrated to bind to all tested bacteria and yeast, and inhibit the replication of SGIV. Overexpression of EcSAA in grouper spleen (GS) cells could also inhibit the replication of SGIV. These results suggest that EcSAA may be an important molecule in the innate immunity of grouper.

1. Introduction

Innate immunity plays an important role in early defense mechanisms and serves to initiate the acquired immune response. The acute phase response (APR) is a complicated and systemic early defense system activated by tissue injury, infection, surgical trauma and inflammation [1,2], which results in a remarkable change in the concentrations of many plasma proteins, known as acute phase proteins (APPs) [3].

The great majority of APPs are synthesized in hepatocytes, also in extra-hepatic sites such as the brain and leukocytes [4]. It responses quickly and becomes a complicated but precise regulation network. APPs play an important role in a variety of the defense-related activities such as killing infectious microbes, repairing tissue damage and restoring healthy (homeostatic) state [5]. Serum amyloid A (SAA) is a major APP in mammals. The studies showed that the gene encoding SAA from trout acts as an effective gene of innate immunity which is known to be regulated by the Toll-like receptor (TLR) signaling cascade. It has also been discussed that SAA may even constitute an endogenous TLR4 ligand [6]. SAA homologs have been identified in all vertebrates investigated and are highly conserved [2]. In recent years, SAA homologs have also been identified and characterized from some fish, such as arctic char (*Salvelinus alpinus*), common carp (*Cyprinus carpio*), Atlantic salmon (*Salmo salar*), zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) [6–10].

The orange-spotted grouper , *Epinephelus coioides*, is widely cultured in China and Southeast Asian countries. As a favorite marine food fish, it is commercially important in live marine fish market. However, in recent years, with rapidly developing marine farming activities, outbreaks of viral diseases have affected grouper aquaculture industry causing heavy economic losses. Singapore grouper iridovirus (SGIV), a novel iridovirus in the genus *Ranavirus*, is one of the major pathogens that resulted in significant economic losses in grouper aquaculture [11,12]. In order to find immune-relevant factors responsible for virus infection, two suppression subtractive hybridization (SSH) libraries from the spleen of SGIV-infected grouper, *E. coioides*, have been described in our laboratory [13]. In our previous

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studies, interleukin enhancer-binding factor 2, C-type lectin, β-defensin, leukocyte cell-derived chemotaxin-2 and hepcidin1/2 have been cloned from *E. coioides*, and results suggested that they may be important molecules involved in pattern recognition and pathogen elimination in the innate immunity of grouper [14–18].

In the present study, the molecular characteristics of grouper SAA, and the tissue distributions, expression patterns after challenging with bacterial and viral pathogens were investigated. The binding activity of the EcSAA and the inhibition of the replication of SGIV were also investigated. These present studies will help us to better understand its innate immune mechanisms in the anti-bacterial or anti-virus response of fishes.

2. Materials and methods

2.1. Fish

Juvenile orange-spotted grouper, *E. coioides* (40–50 g) were purchased from a mari-culture farm at Daya bay, Huizhou City, Guangdong Province, China. After maintenance in aerated flow-through seawater for 3 days, these fish were used for the challenge experiments.

2.2. Preparation of microbial cells and SGIV

Vibrio vulnificus, *Vibrio parahaemolyticus*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Saccharomyces cerevisiae* (American Type Culture Collection (ATCC) 9763), *Staphylococcus aureus* (ATCC 12598), *Vibrio alginolyticus* and *Escherichia coli* JM109 were obtained from our laboratory. *V. parahaemolyticus* was cultured with a TCBS agar plate at 26 °C, while *V. vulnificus* was cultured at 26 °C with aeration in Luria–Bertani (LB) medium prepared with fresh seawater. *S. cerevisiae* were cultured with 2 × YPD medium with 3% glucose (4% bactotryptone, 2% bacto-yeast extract (pH 5.8)) at 30 °C. Other bacterial strains were cultured at 37 °C in LB prepared with the distilled water. All microbial strains were harvested by centrifugation at 3500 g for 10 min and suspended in the buffer for an appropriate concentration. Quantification was performed by plating various bacteria dilutions on agar plates.

Cell lines of grouper spleen (GS) were propagated by the recommended methods with Leibovitz's L15 culture medium with 10% fetal calf serum.

Propagation of SGIV was performed as described previously [12]. The viral titer of SGIV was 10^5 $TCID_{50/ml}$.

2.3. Immunization experiments

In bacteria challenging experiment, each control and challenged sample was injected with 100 μl PBS and a live microbial PBS suspension (10^5 CFU/μl), respectively. Livers of six fish in each group were collected for quantitative real-time PCR (qRT-PCR) at 4, 8, 12, 24, 36 and 48 h for bacteria-challenged groups.

In SGIV challenging experiment, each control and challenged sample was injected 50 μl PBS and SGIV at a concentration of 10^5 $TCID_{50/ml}$, respectively. Livers of six fish in each group were collected for qRT-PCR at 4, 8, 12, 24, 36, 48 and 72 h for SGIV-challenged groups.

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated from different tissues of grouper, *E. coioides*, using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. Each of the samples contained 6 independent individuals respectively to eliminate the individual differences. The RNA was treated with RQ1 RNase-Free DNase (Promega, USA) to remove contaminated DNA. The quality of total RNA was assessed by electrophoresis on 1% agarose gel. Total RNA was reverse transcribed to synthesize the first-strand cDNA by ReverTra Ace kit (TOYOBO, Japan) according to the manufacturer's instructions.

2.5. Cloning and sequence analysis of E. coioides serum amyloid A (EcSAA) cDNA and genomic DNA

The first-strand cDNA was synthesized from the total liver RNA with the SMART™ RACE cDNA amplification kit (Clontech) for 3′ RACE and 5′RACE. Two primers F1 (GAGAGGAGCAGGGGGCAGATGGG) and R1 (AGCCCATCTGCCCCCTGCTCCTC) were designed based on the identified EST sequence of SAA (GH612458). PCR was performed with 10 μM F1 or R1 and 500 nM of Nested Universal Primer A (NUP, Clontech). Denaturation was performed at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s.

Genomic DNA was extracted from liver of orange-spotted grouper using the tissue Genomic DNA Purification System (Sino-American Biotechnology Co., China), according to the manufacturer's instructions. The primers F2 (TCATAAGATTCTCTACGATCTATCA) and R2 (GTTGATGACTTTCATACTAATATT) were designed according to the 5-untranslated region (UTR) and 3-UTR of the full-length cDNA of EcSAA. 25 ng of genomic DNA was used for the genomic PCR with an LA Taq (TaKaRa) using F2 and R2. PCR was performed with an initial denaturation step of 5 min at 94 °C, and then 35 cycles were run as follows: 94 °C 45 s, 55 °C 45 s, 72 °C 1.5 min, and 72 °C elongation for 5 min.

2.6. TA cloning, sequencing and database analysis

PCR products were analyzed on 1% agarose gels, extracted with an AxyPrep DNA gel extraction kit (AxyGEN), and then ligated into pMD18-T vectors (TaKaRa) and transformed into competent *E. coli* DH5 cells. Positive colonies were screened by PCR and at least two recombinant plasmids were sequenced.

Sequences were analyzed based on nucleotide and protein databases using the BLASTN and BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST/). The protein and its topology prediction were performed using software at the ExPASy Molecular Biology Server (http://expasy.pku.edu.cn). Multiple sequence alignment of the EcSAA was performed with the Clustal X multiple-alignment software. MEGA 4.0 was also used to produce the phylogenetic tree. Neighbor-joining (NJ) method was used for the phylogenetic analysis. One thousand bootstraps were selected for the NJ trees to check its repeatability.

2.7. Analysis of EcSAA mRNA expression profiles

qRT-PCR was employed to detect the EcSAA expression profiles using β-actin as a reference gene. The qRT-PCR primers, F3 (ATTGCTCTGATTCTCATTGTGG)/R3 (CATCGTAGTTTCCTCTGG) and actin-F (TACGAGCTGCCTGACGGACA)/actin-R (GGCTGTGATCTCCTTTTGCA), were designed based on the full-length cDNA of EcSAA and β-actin. qRT-PCR was performed on Roche LightCycler 480 Real-time PCR system (Roche, Switzerland) using the 2 × SYBR Green Real-time PCR Mix (TOYOBO, Japan). PCR amplification was performed in triplicate wells, using the cycling parameters: 94 °C for 5 min, followed by 40 cycles of 5 s at 94 °C, 10 s at 60 °C and 15 s at 72 °C. Relative gene expression was analyzed by the comparative Ct method ($2^{-\triangle\triangle C_T}$ method). Target C_T values were normalized to the endogenous gene β-actin. Results for each treated sample were expressed as N-fold changes in target gene expression relative to the same gene target in the calibrator sample, both normalized to the β-actin gene. All samples were analyzed in three duplications and all data were given in term of relative mRNA expression level as means ± SD, and then subjected to Student's *t-test*. Differences were considered significant at $p < 0.05$ or $p < 0.01$.