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Short communication

Isolation and function analysis of apolipoprotein A-I gene response to virus infection in grouper

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ABSTRACT

Apolipoproteins, synthesized mainly in liver and intestine and bounded to lipids, play important roles in lipid transport and uptake through the circulation system. In this study, an apolipoprotein A-I gene homologue was cloned from orange-spotted grouper *Epinephelus coioides* (designed as Ec-ApoA-I) by rapid amplification of cDNA ends (RACE) PCR. The full-length cDNA of Ec-ApoA-I was comprised of 1278 bp with a 792 bp open reading frame (ORF) that encodes a putative protein of 264 amino acids. Quantitative real-time PCR (qPCR) analysis revealed that Ec-ApoA-I was abundant in liver and intestine, and the expression in liver was significantly ($P < 0.01$) up-regulated after the stimulation of LPS, Poly(I:C), *Vibrio alginolyticus*, and Singapore grouper iridovirus (SGIV). Recombinant Ec-ApoA-I (rEc-ApoA-I) was produced in *Escherichia coli* BL21 (DE3) expression system exhibited bacteriolyticactivity against *Micrococcus lysodeikticus* and *Aeromonas hydrophila*. Intracellular localization revealed that Ec-ApoA-I distributed in both cytoplasm and nucleus, and predominantly in the cytoplasm. Overexpression of Ec-ApoA-I in grouper Brain (GB) cells could inhibit the replication of SGIV. These results together indicated that Ec-ApoA-I perhaps is involved in the responses to bacterial and viral challenge.

1. Introduction

Fishes have a number of constitutive and inducible innate immune responses to defend against infection, many of which are similar to those of mammals. Some of the different soluble mediators include: pro-inflammatory cytokines, complement, and antimicrobial peptides [1]. Teleosts possess many of the same pro-inflammatory cytokines and complement components as mammals. Compared to mammals, teleosts possess a smaller number of antimicrobial peptides [2]. However, many species of fishes have other innate defense molecules such as natural antibodies [3,4], apolipoproteins [5,6], lysozyme [7–9], larger nonpeptide antimicrobial compounds such as squalamine [10], and other cationic steroidal derivatives [11].

Apolipoproteins, the protein component of various plasma lipoproteins, synthesized mainly in liver and intestine and bounded to lipids, play important roles in lipid transport and uptake through the circulation system [12]. A great deal of attention had been focused on lipoproteins and apolipoproteins in humans ever since the relationship between specific lipoproteins and cardiovascular disease became apparent [13]. However, little information has been reported to apolipoproteins in lower vertebrates [13]. Apolipoprotein A-I (apoA-I) is the major protein component of high density lipoprotein (HDL) particles in serum and participates in the reverse transport of cholesterol from tissues to the liver for excretion as the major activator of lecithin: cholesterol acyltransferase (LCAT) [14]. Since most fish utilize lipids as the major energy source in contrast to mammals which mainly use carbohydrates [15], lipid metabolism appears more important for homeostasis maintenance in fish than that in homeotherms [16].

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Several other functions have been attributed to apoA-I. ApoA-I has a variety of immune-related properties including inhibition of bacterial endotoxin, antiviral activity, and inhibition of inflammatory cytokines. Mammalian ApoA-I demonstrates direct antimicrobial activity against a variety of viruses and bacteria [2,7,17–25]. For example, human ApoA-I has antiviral activity against Herpes Simplex Virus (HSV), human immunodeficiency virus (HIV), and xenotropic murine virus [21–23]. Teleostean ApoA-I isolated from carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), Channel Catfish (*Ictalurus punctatus*) and striped bass (*Morone saxatilis*) demonstrates in vitro antibacterial activity [2,18,24,25].

Groupers, *Epinephelus* sp., are widely cultured in China and Southeast Asian countries. The emergence of bacterial and viral pathogens, including *Vibrio alginolyticus*, iridovirus and nervous necrosis virus, caused heavy economic losses in grouper aquaculture [26–28]. For example, the gram-negative bacterium, *V. alginolyticus*, has frequently been identified as the pathogen responsible for the infectious disease called vibriosis. This disease is one of the major challenges facing grouper aquaculture, which has caused substantial economic losses globally [28]. Further, Singapore grouper iridovirus (SGIV) was isolated from diseased groupers, which belonged to family *Iridoviridae* [26,29]. SGIV caused serious systemic diseases and resulted in more than 90% mortality in grouper either in fish farm or challenge experiments. The virus infection is characterized as hemorrhage and enlargement of the spleen of infected fish [29].

In the present study, the molecular characteristics of grouper ApoA-I, the tissue distribution, and expression patterns were investigated. The recombinant Ec-ApoA-I protein and mouse anti-Ec-ApoA-I were obtained. The intracellular localization and the antiviral activity were also studied. These present studies will help us for better understanding of 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, *Epinephelus coioides*(40–50 g) were purchased from a marin-culture farm at Honghai bay, Shanwei City, Guangdong Province, China. After maintenance in aerated flow-through seawater for 7 days, groupers were used for the challenge experiments.

2.2. Preparation of bacterial cells and virus

V. alginolyticus was obtained from our laboratory. *Micrococcus lysodeikticus*, and *Aeromonas hydrophila* were kindly provided by Professor Yongcan Zhou, Hainan University. All bacterial strains were cultured at 37 °C for 24 h in Luria–Bertani (LB) prepared with the distilled water, then harvested by centrifugation at 3500 g for 10 min and suspended in PBS for an appropriate concentration. Quantification was performed by plating various bacteria dilutions on agar plates.

Cell lines of grouper spleen (GS) and grouper brain (GB) were propagated by the recommended methods with Leibovitz's L15 with 10% fetal calf serum at 28 °C [30].

Propagation of SGIV was performed as described previously [29]. The viral titer of SGIV was 10^5 $TCID_{50}$/ml.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from the liver of groupers, using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. Each of the samples contained 6 independent individuals to eliminate 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.

2.4. EST analysis and cloning of the full-length Ec-ApoA-I cDNA

EST sequences were obtained from two spleen-complementary DNA libraries which were constructed from SGIV-infected and PBS-injected grouper [31]. BLAST analysis of all the EST sequences revealed that one EST (no. 63681111, 506 bp) was homologous to previously identified Apolipoprotein A-I (ApoA-I). Based on the EST sequence, two specific primers, F1 and R1 (Table 1) were designed to clone the full sequence of Ec-ApoA-I by rapid amplification of cDNA ends (RACE) approach. 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. 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 *Escherichia coli* DH5 cells. The sequencing results were verified and subjected to cluster analysis.

2.5. Sequence analysis

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 Ec-ApoA-I was performed with the Clustal X multiple-alignment software. Neighbor-joining (NJ) method implemented in MEGA 4.0 was used for phylogenetic analysis [32]. The robustness of bifurcations was estimated with bootstrap analysis and bootstrap percentages were obtained with 1000 replicates.

2.6. Real-time PCR analysis of Ec-ApoA-I mRNA expression in different tissues

Total RNA from liver, spleen, kidney, brain, intestine, heart, skin, muscle, stomach, gill and head kidney were extracted from six adult groupers as parallel samples using TRIzol reagent

Table 1
Primers used in the present study.

Primer	Sequence (5′–3′)
F1	GCCCATCTTCAATGAGTATTCCACC
R1	GCCTGGTGGAATACTCATTGAAGAT
F2	TCTTCAATGAGTATTCCACCAGG
R2	GCTTCCACTATGGGTGTCAGG
F3	GCGGATCCATGAAATTTGTGGCTCTTGCTCTCG
R3	GCGAATTCTTATTGCTTGTTGACGGTTT
SGIV ORF049-F	CCCGCAATGAACTCGCCAAAACT
SGIV ORF049-R	CCGTGACGTACTGCCAAGCCTGA
SGIV ORF072-F	GCACGCTTCTCTCACCTTCA
SGIV ORF072-R	AACGGCAACGGGAGCACTA
SGIV ORF086-F	ATCGGATCTACGTGGTTGG
SGIV ORF086-R	CCGTCGTCGGTGTCTATTC
ISG15-F	CCTATGACATCAAAGCTGACGAGAC
ISG15-R	GTGCTGTTGGCAGTGACGTTGTAGT
Mx-I-F	CGAAAGTACCGTGGACGAGAA
Mx-I-R	TGTTTGATCTGCTCCTTGACCAT
Actin-F	GGCATCACACCTTCTACAACGAG
Actin-R	AGAGGCATACAGGGACAGCACAG