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Short communication

Identification of a retinoic acid-inducible gene I from Japanese eel (*Anguilla japonica*) and expression analysis *in vivo* and *in vitro*

Jianjun Feng [a, b, c, *], Songlin Guo [a, b, c], Peng Lin [a, b, c], Yilei Wang [a, b, c], Ziping Zhang [d], Zaipeng Zhang [a, b, c], Lili Yu [a, b, c]

[a] College of Fisheries, Jimei University, Xiamen, 361021, Fujian Province, China
[b] Engineer Research Center of Eel Modern Industry Technology, Ministry of Education, China
[c] Key Laboratory of Healthy Mariculture for the East China Sea, Ministry of Agriculture, China
[d] College of Animal Science, Fujian Agriculture and Forestry University, Fuzhou, 350002, China

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ABSTRACT

RIG-I (retinoic acid inducible gene-I) is one of the key cytosolic pattern recognition receptors (PRRs) for the recognition of cytosolic viral nucleic acids and the production of type I interferons (IFNs). The full-length cDNA sequence of RIG-I (*Aj*RIG-I) in Japanese eel (*Anguilla japonica*) was identified and characterized in this article. The full-length cDNA of *Aj*RIG-I was 3468 bp, including a 5′-untranslated region (UTR) of 52 bp, a 3′-UTR of 617 bp and an open reading frame (ORF) of 2799 bp encoding a polypeptide of 933 amino acid residues with a calculated molecular mass of 106.2 kDa. NCBI CDD analysis showed that the *Aj*RIG-I protein had the typical conserved domains, including two adjacent caspase activation and recruitment domains (CARDs), a DEXDc domain, a HELICc domain and a C-terminal regulatory domain (RD). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis revealed a broad expression for *Aj*RIG-I in a wide range of tissues, with the predominant expression in liver, followed by the gills, spleen, kidney, intestine, skin, and the very low expression in muscle and heart. The *Aj*RIG-I expressions in liver, spleen and kidney were significantly induced following injection with LPS, the viral mimic poly I:C, and *Aeromonas hydrophila* infection. *In vitro*, the *Aj*RIG-I transcripts of Japanese eel liver cells were significantly enhanced by poly I:C and PGN stimulation, down-regulated with CpG-DNA treatment whereas no change of the expression level was found post LPS challenge. These results collectively suggested *Aj*RIG-I transcripts expression possibly play an important role in fish defense against viral and bacterial infection.

1. Introduction

The cell signaling of the innate immune response is characterized by its germline-encoded pattern recognition receptors (PRRs), which sense the conserved molecular structure of a pathogen (PAMPs), such as LPS, peptidoglycans (PGN), DNA motif, double strand RNA (dsRNA), single strand RNA (ssRNA), and poly I:C, et al. The germline-encoded PRRs currently are classified into five major groups including the well-known Toll-like receptors (TLRs), the C-type lectins receptors (CLRs), retinoic acid inducible gene I (RIGI)-like receptors (RLRs), the nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs; previously designated as the nucleotide binding oligomerization domain (NOD)-like receptors), and the newly classified family of HIN200/PYHIN family members that have recently been designated absent in melanoma (AIM)-like receptors (ALRs) [1].

To date, three RLR members have been identified: RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation associated factor 5), and LGP2 (laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2) [2]. Among them, RIG-I has been shown to play a key role to antiviral immunity [3]. The structures of RIG-I possess one or two caspase recruitment domains (CARD) at their N-terminus, followed by a DEAD-like C-terminal helicase domain (DEXDc), a helicase conserved C-terminal domain (HELICc), and a C-terminal domain (CTD, also called repressor domain, (RD) [1]. The RNA helicase domain and RD provide their ability to discriminate and bind viral derived nucleic acids, whereas the

* Corresponding author. College of Fisheries, Jimei University, Xiamen, 361021, Fujian Province, China.
E-mail address: 2394417698@qq.com (J. Feng).

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CARDs can transmit a signal via the recruitment of downstream molecules [4]. In the absence of dsRNA, the intramolecular interaction between the RD and CARD domains could maintain the conformation of RIG-I in resting state and its activity was inhibited [5]. Once RNA virus entered into cells, the intracellular dsRNA from virus itself or the replication of virus were first binded to the RD and subsequently with the helicase. Such interactions initiated the reorientation of the pincer domain and then leaded to ATP hydrolysis and release of the N-terminal CARDs containing signaling module from the repressed conformation [6]. The molecular interaction between the released N-terminal CARDs of RIG-I and the CARD domain of an adaptor protein—the mitochondrial antiviral signaling protein, MAVS (also known as Cardif, VISA and IPS-1) triggered the activation of the IPS-1 signalosome include interferon regulatory factor 3 (IRF3), IRF7, and NF-kB [7]. These processes result in a signaling cascade that culminates in IFN production and expression of proteins with direct antiviral or immune-modulating activities to control virus infection [8].

RIG-I was initially characterized as a dsRNA-binding protein that triggered IFN induction and virus signaling in response to the synthetic dsRNA poly I:C [8]. The subsequent studies have shown that RIG-I plays a key role in recognizing RNA viruses [9–13]. Furthermore, several DNA viruses have now been shown to activate this pathway such as herpes-simplex virus-1 [14], Adenovirus, Epstein-Barr virus [15], hepatitis B virus [16] and Vaccinia virus [17]. Surprisingly, both of the intracellular gram negative and positive bacterium including *Legionella pnuemophila* and *listeria monocytogenes* were shown to activate type I IFN responses through RIG-I signaling [18,19]. Moreover, the bacterial lipopolysaccharide has been found to induce the expression of RIG-I in both macrophages and vascular endothelial cells [20,21]. Thus, the regulatory functions of RIG-I are strikingly broad, playing a role not only in antiviral responses but in antibacterial responses as well.

In teleost fish, several RIG-I genes have been cloned and identified in Atlantic salmon (*Salmo salar*) [22], zebrafish (*Danio rerio*) [9], channel catfish (*Ictalurus punctatus*) [23], and grass carp (*Ctenopharyngodon idella*) [24] demonstrating that fish possess a functional RIG-I pathway in host innate immune defense against both dsRNA viruses and ssRNA viruses. Following bacterial infection, the RIG-I gene channel catfish and zebrafish have been shown to increase significantly which indicated the RIG-I genes were involved in anti-bacterial immune response [9,23]. However, the orthologues of RIG-I sequences have been reported not appear in some model fish species such as fugu (*Takifugu rubripes*), Japanese flounder (*Paralichthys olivaceus*), large yellow croaker (*Larimichthys crocea*), and rainbow trout (*Oncorhynchus mykiss*), demonstrating the functional RIG-I genes have been lost in some teleost fish species [4,25]. Therefore, the role of RIG-I both in antiviral and anti-bacterial innate immune is waiting for further studied in more fishes.

Japanese eel (*Anguilla japonica*), one of the most crucial economic species for aquaculture in Asia [26], has suffered from serious diseases caused by viral, bacterial, and parasitic infection in recent years that resulted in huge economic losses [27]. Better understanding of the antiviral and the anti-bacterial immune mechanisms may contribute to the development of management strategies for disease control and long term sustainability of eel farming. This study is the first to describe the Japanese eel's full-length cDNA of *Aj*RIG-I along with its expression pattern in various organs. Next, we evaluated the temporal expression profiles of the gene in liver, spleen, and kidney after stimulation with LPS, poly I:C, and *Aeromonas hydrophila* at 0, 6, 12, 24, 48, and 72 h. Furthermore, the expression pattern of *Aj*RIG-I was examined in Japanese eel liver cells in response to the different PARMs, including poly I:C, LPS, CpG-DNA and PGN at 0, 3, 6, 12, 24, and 48 h. The information presented in this study will facilitate research in the innate immune responses of Japanese eel upon both virus and bacteria infection.

2. Materials and methods

2.1. Fish collection and immune challenge

Healthy Japanese eels (*Anguilla japonica*), weighing 45–50 g, were purchased from an eel farm (Fuqing, China). They were kept in a 1000-L tank at 25 °C with recirculated and aerated water for a week to acclimate to laboratory conditions. Liver, spleen, gills, kidney, intestine, heart, skin, and muscle were harvested and then frozen in liquid nitrogen and then stored at −80 °C for RNA extraction.

Aeromonas hydrophila were inoculated in Tryptone soya broth (TSB) and incubated on a shaker at 28 °C for 24 h [27]. The bacteria were collected and diluted to the concentration of 4×10^4-cfu/mL in 0.01 mmol/L PBS (pH = 7.4). Fish immune stimulation was performed by intraperitoneal injection of 250-μL LPS (Sigma, 4 mg/mL) in phosphate buffered saline (PBS), 250-μL poly I:C (Sigma, 2 mg/mL) in PBS, and 250-μL 4×10^4 cfu/mL *A. hydrophila* in PBS, respectively. Fish injected with 250 μL PBS were used as controls. Four fish were sacrificed for the control group and experimental group for each time point. Liver, spleen and kidney of each group were collected at 0, 6, 12, 24, 48, and 72 h after injection and preserved for Quantitative real-time polymerase chain reaction (qRT-PCR).

2.2. Cell culture and treatments

For *in vitro* studies, Japanese eel liver cells prepared in our lab [28] was cultured and maintained in DMEM medium with 16.7% fetal bovine serum (FBS, Sigma) and antibiotics (100 IU/mL penicillin and100 μg/mL Gibberellin, Gibco) at 27 °C with 5% CO_2. Japanese eel liver cells were seeded in 60 mm culture dishes and at about 90% confluence the day before the treatment. Cells were then treated with 30 μg/mL LPS (Sigma), 50 μg/mL poly I:C (Sigma), 30 μg/mL CpG-DNA (Sangon Biotech, Shanghai), 30 μg/mL PGN (Sigma) and the untreated cells were served as control. Four parallel samples were included of each group at 0, 3, 6, 12, 24, and 48 h after treatment.

2.3. Cloning of full-length cDNA of AjRIG-I

Total RNA from tissues was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. Total RNA from cells was isolated using E.Z.N.A.™ Total RNA Kit II (Omega) following manufacturer's instructions. Total RNA from the liver of Japanese eel was used to synthesize the first-strand cDNA for the RACE reaction using the SMART RACE cDNA Amplification Kit (Takara, China) according to the manufacturer's instructions. Primers were designed according to the partial sequence of RIG-I from the Japanese eel transcriptome database in our lab with a local version of the Primer Premier 5.0 design software tool (http://www.premierbiosoft.com/primerdesign/index.html) (Supplementary Table 1) and PCR was performed to amplify the partial cDNA sequences of eel RIG-Igene. The purified PCR product was inserted into the pMD19-T vector (Takara, China) and transformed into JM109 competent cells. The plasmids from positive clones were subjected to DNA sequencing by Sangon Biotech Corp (Shanghai, China). Based on the partial gene sequence of RIG-I, the full-length cDNA sequence was pulled out using 5′and 3′ RACE System for Rapid Amplification of cDNA Ends (Takara, China) with gene-specific primers as listed in Supplementary Table 1. The RACE