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Identification and functional characterization of an interferon regulatory factor 7-like (IRF7-like) gene from orange-spotted grouper, *Epinephelus coioides*

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ABSTRACT

Interferon regulatory factor (IRF) 7 plays a crucial role in modulating cellular responses to viral infection and cytokines, including interferons (IFNs). In the present study, a novel IRF7 gene (designated as EcIRF7) was cloned and characterized from orange-spotted grouper, *Epinephelus coioides*. The full-length EcIRF7 cDNA is composed of 2089 bp and encodes a polypeptide of 433 amino acids with 81% identity to IRF7 of *Siniperca chuatsi*, and the genomic DNA of EcIRF7 consists of 9 exons and 8 introns, with a length of approximately 5629 bp. EcIRF7 contains three conserved domains including a DNA-binding domain (DBD), an IRF associated domain (IAD) and a serine-rich domain, all of which are highly conserved across species. Recombinant EcIRF7 was expressed in *Escherichia coli* BL21 (DE3) and purified for mouse anti-EcIRF7 serum preparation. Realtime quantitative PCR (RT-qPCR) analysis revealed a broad expression of EcIRF7, with a relative strong expression in spleen, kidney, skin and intestine. The expression of EcIRF7 was differentially up-regulated after stimulation with *Vibrio vulnificus*, *Staphylococcus aureus* and Singapore grouper iridovirus (SGIV). EcIRF7 showed similar intracellular localization pattern to those of mammalian and chicken, and translocated into nucleus after SGIV infection. Further more, EcIRF7 was proved to be capable of activating zebrafish type I IFN promoter and inhibiting the replication of SGIV in grouper spleen (GS) cells. These results suggest that EcIRF7 is potentially involved in grouper immune responses to invasion of viral and bacterial pathogens.

1. Introduction

The innate immunity provides immediate defense against microbial invasion in invertebrate and vertebrate animals (Aderem and Ulevitch, 2000; Bieniasz, 2004; Medzhitov and Janeway, 1997). In mammals, interferon (IFN) regulatory factors (IRFs) are a family of transcription mediators that have been initially identified to induce the expression of IFNs and IFN-stimulated genes (ISGs), all of which play central roles in initiating host innate immune responses to virus and bacteria infection (Mamane et al., 1999; Taniguchi et al., 2001; Tamura et al., 2008; Fujita et al., 1988). In recent years, with the discovery of pattern recognition receptors (PRRs), including the transmembrane toll-like receptors (TLRs), and the cytosolic PRRs (like RIG-I, MDA5, PKR and NOD proteins), IRFs gained much more attentions in activating immune cells and coordinating innate and adaptive immune responses (Honda and Taniguchi, 2006; Honda et al., 2006). IRFs have been believed to play diverse roles in initiating antiviral responses, regulating inflammatory cytokine expression, controlling the cell-cycle and apoptosis, mediating the development of macrophages, dendritic cells, B and T lymphocytes (Honda and Taniguchi, 2006; Paun and Pitha, 2007; Gabriele and Ozato, 2007; Brüstle et al., 2007).

To date, nine human cellular IRF members (IRF-1 to -9), and three virus-encoded homologues have been identified (Nguyen et al., 1997; Barnes et al., 2002). All IRFs share significant homology in the N-terminal ~120 amino acids region, which contains a well-conserved DNA-binding domain (DBD) as well as a five tryptophan repeat cluster, which is responsible for the binding to GAAA or AANNGAA motifs existing in the promoters of type I IFNs and ISGs (Paun and Pitha, 2007). In addition, all IRFs but IRF1 and IRF2, contain the IRF associated domain (IAD) at the C terminus mediating the formation of homodimers or heterodimers with other IRFs and other transcription factors to target promoters (Eroshkin and Mushegian, 1999; Hiscott, 2007a). Among the mammalian IRFs, the IRF3 subfamily, including the IRF3 and IRF7, which are highly homologous, have been implicated to be crucial transducers of virus mediated IFN signaling (Honda and Taniguchi, 2006; Paun and Pitha, 2007). In mammalian cells, virus-induced type I IFNs response is triggered by sensing the nucleic acids of virus through two classes of cellular PRRs: Toll-like receptors (TLR-3, -7, -8, and -9) and cytosolic PRRs

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(RIG-I, MDA5 and DAI) (Kawai and Akira, 2008; Wang et al., 2008). All these events trigger signaling cascades and lead to the activation of IRF3 and IRF7, and finally activate the promoter of target IFN genes, as well as some ISGs (Fitzgerald et al., 2003; Hiscott, 2007b; Chau et al., 2008).

IRF7 was initially identified as a repressor binding to the *Bam*H I Q promoter (Qp) of the Epstein-Barr virus (EBV) in type III latent infection state, and a splice variant of IRF7 (IRF7H) was characterized as an activator of IFNα promoter and played a critical role in regulating the IFNα gene expression in plasmacytoid dendritic cells (pDCs) (Zhang and Pagano, 1997; Au et al., 1998). Unlike IRF3, which has a constitutively expression both in immune and non-immune cells, the constitutively expression of IRF7 is limited to some lymphoid cells, especially in pDCs. Meanwhile, unlike IRF3, the IRF7 expression could be strongly induced by extracellular stimulus, such as viral infection, type I IFNs, TNF-α, LPS and so on (Honda and Taniguchi, 2006). In resting cells, IRF3 and IRF7 are located in the cytoplasm, while after virus stimulation, these factors undergo serine residues phosphorylation of their C-terminal serine-rich conserved domain, allowing them to dimerize and translocate into the nucleus, where these dimmers initiate gene transcription by binding to the target promoter regions (Marie et al., 1998; Lin et al., 2000). Recently, more and more evidences demonstrated IRF7 as a "master regulator" of both IFNα and IFNβ expression in pDCs, while IRF3 was considered mainly responsible for the initial induction of the IFNβ gene (Marie et al., 1998; Sato et al., 1998; Sato et al., 2000; Honda et al., 2005; Honda and Taniguchi, 2006). Furthermore, IRF7 is also an ISG, which could be up-regulated by early phase IFNs. After the initial induction of type I IFNs, the positive-feedback regulation comes into effect through the Jak-STAT pathway, and the participation of type I IFN-induced IRF7 is crucial for the induction of type I IFN genes to occur in full (Platanias, 2005; Honda and Taniguchi, 2006).

Teleost fish, as a group of vertebrates, are reported to possess IFN systems which represents the first line of innate immune defense against infective bacterial- and viral-agents (Ellis, 2001; Larsen et al., 2004; Robertsen, 2006; McBeath et al., 2007). Until now, a variety of IRF genes have been cloned from fish species: IRF1 and IRF2 from Japanese flounder, rainbow trout, Crucian carp, sea bream and so on (Yabu et al., 1998; Collet et al., 2003; Ordas et al., 2006; Shi et al., 2007; Sun et al., 2007), IRF3 from Atlantic salmon and rainbow trout (Holland et al., 2008; Bergan et al., 2010), IRF4 and IRF8 from rainbow trout (Holland et al., 2010). Recently, IRF7-like genes have also been identified and characterized in Atlantic salmon, rainbow trout, Crucian carp, mandarin fish and snakehead fish (Holland et al., 2008; Kileng et al., 2009; Bergan et al., 2010; Zhang et al., 2003; Sun et al., 2007; Jia and Guo, 2008). However, the roles of IRF7 in fish immune responses, especially in responding to virus infection, have not been elucidated yet.

Orange-spotted grouper, *Epinephelus coioides*, one of the major species being maricultured in China and Southeast Asian countries, are high-priced and popular seafood fish. In recent years, with the rapid development of intensive marine fish farming, viral pathogens, particular the iridoviruses, like Singapore grouper iridovirus (SGIV), have affected the grouper aquaculture industry causing heavy economic losses (Qin et al., 2003; Song et al., 2004). However, in comparison with other high commercially important fish, the information of immune-related genes of grouper is scarce. The better understanding of IFN system in grouper will be helpful to the control of viral diseases. Up to now, only IRF1- and IRF2-like genes have been cloned in grouper, *Epinephelus* sp. (Shi et al., 2010).

In the present study, the full-length cDNA and genomic DNA of IRF7 (EcIRF7) were cloned from *E. coioides*. The intracellular localization and the temporal expression profiles of EcIRF7 were investigated. In particular, the transcriptional activation activity of EcIIRF7 on zebrafish type I IFN promoter was studied by luciferase reporter assay, and the anti-virus effect of EcIRF7 against SGIV infection was analyzed in grouper spleen (GS) cells.

2. Materials and methods

2.1. Fish and cell lines

Juvenile orange-spotted grouper, *E. coioides*, about 50 g in body weight, 6 months old, were purchased from a fish farm in Zhanjiang, Guangdong province, China. Fish were maintained in a laboratory recirculating seawater system at 25–30 °C for 2 weeks. The fish were anesthetized with tricaine methanesulfonate (MS222) (100 mg/L, Sigma, USA) before killing. A series of tissue samples including heart, liver, spleen, intestine, brain, skin, muscle and stomach were dissected from the killed fish and immediately frozen by liquid nitrogen, followed by storage at −80 °C until used.

GS cells, derived from the spleen of orange-spotted grouper, *E. coioides* were grown in Leibovitz's L-15 medium containing with 10% fetal bovine serum (FBS) (Gibco, USA) at 27 °C (Qin et al., 2006).

2.2. Bacteria and Singapore grouper iridovirus (SGIV) challenge of the fish

Vibrio vulnificus, originally isolated from diseased grouper, *E. coioides* was cultured at 26 °C with aeration in Luria-Bertani (LB) medium prepared with fresh seawater. *Staphyloccocus aureus* was obtained from American Type Culture Collection (ATCC 12598) and cultured at 37 °C in LB medium prepared with distilled water. The microbial strains were harvested by centrifugation at 3500 × *g* for 10 min and suspended in the phosphate buffered saline (PBS) for an appropriate concentration. Quantification was performed by plating various bacteria dilutions on agar plates. SGIV was first isolated from the brown-spotted grouper, *Epinephelus tauvina*, and the propagation of SGIV was performed as described previously (Qin et al., 2003). Briefly, GP cells derived from the brown-spotted grouper, *E. tauvina* were cultured in Eagle's minimum essential medium containing 10% fetal bovine serum at 25 °C. Virus was inoculated onto confluent monolayer of the GP cell cultures at a multiplicity of infection (MOI) of approximately 0.1. When the cytopathogenic effect (CPE) was sufficient, the medium containing SGIV was harvested and centrifuged at 3000 × *g* for 10 min at 4 °C, and then the supernatant was collected as the SGIV solution and stored at −80 °C until used.

The *in vivo* pathogenicity experiments were performed by intraperitoneal injection (i.p.). Before injection, fish of each group were anesthetized with MS222. In bacteria challenging experiment, each control and challenged sample was injected with 100 μl PBS and a live microbial PBS suspension (1×10^9 CFU/ml), respectively. In SGIV challenging experiment, each control and challenged sample was injected with 50 μl PBS and SGIV at a concentration of 1×10^5 $TCID_{50}$/ml, respectively. All the infectious doses of these stimulus used in *vivo* challenge experiments were optimized by our previous studies and were less than lethal doses (Wei et al., 2010). Grouper spleen of 5 fish in each group were collected at different time points of post-injection and immediately frozen by liquid nitrogen, followed by storage at −80 °C until used for RNA extraction.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol. The quality of the RNA was assessed by electrophoresis on 1.0% agarose gel. RNA samples were treated with DNase I to remove contaminated genomic DNA using the Turbo DNA-free kit (Ambion, USA). Total RNA was reverse