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Identification of orange-spotted grouper (*Epinephelus coioides*) interferon regulatory factor 3 involved in antiviral immune response against fish RNA virus

Youhua Huang, Xiaohong Huang, Jia Cai, Zhengliang OuYang, Shina Wei, Jingguang Wei, Qiwei Qin*

Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China

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A B S T R A C T

Interferon regulatory factor 3 (IRF3) is an important transcription factor which regulates the expression of interferon (IFN) and IFN-stimulated genes (ISGs) following virus recognition. In this study, a novel IRF3 gene was cloned from grouper *Epinephelus coioides* (EcIRF3) and its effects against Singapore grouper iridovirus (SGIV) and red spotted grouper nervous necrosis virus (RGNNV) was investigated. The full-length of EcIRF3 cDNA was composed of 2513 bp and encoded a polypeptide of 458 amino acids which shared 82% identity with European seabass (*Dicentrarchus labrax*). EcIRF3 contained three conserved domains including a DNA-binding domain (DBD), an IRF associated domain (IAD) and a serine-rich domain. Expression profile analysis revealed that EcIRF3 was abundant in head kidney, kidney, spleen and gill. Upon different stimuli *in vitro*, the transcript of EcIRF3 was significantly up-regulated after RGNNV infection or treatment with polyinosin-polycytidylic acid (poly I:C). During SGIV infection, the increase of the EcIRF3 transcription was only detected at the late stage, suggesting that EcIRF3 was differently regulated by different stimuli. Immune fluorescence assay indicated that the fluorescence signal of EcIRF3 was increased significantly after infection with RGNNV or treatment with poly I:C, but moderately at the late stage of SGIV infection. Reporter gene assay showed that EcIRF3 activated zebrafish type I IFN and type III IFN promoter *in vitro*. The viral gene transcription and virus production of RGNNV were significantly decreased in EcIRF3 overexpressing cells. However, the ectopic expression of EcIRF3 did not affect the gene transcription and virus production of SGIV. Moreover, the mRNA expression levels of type I IFN and IFN-inducible genes (MxI, ISG15 and ISG56) were increased in RGNNV infected EcIRF3 overexpressing cells compared to empty vector transfected cells. Together, our results demonstrated that IFN immune response mediated by grouper IRF3 was exerted crucial roles for fish RNA virus, but not for DNA virus replication.

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1. Introduction

The innate immunity system constitutes the first line of defense against invading microbial pathogens and is evolutionarily conserved for animals and plants for protecting themselves from these invading threats. In mammals, the innate antiviral mechanism for most cells involves the actions of type I interferon, leading to the induction of IFN relative gene expression [1–3]. The induction of type I interferon is primarily regulated at the transcriptional level, wherein a family of transcription factors, interferon regulatory factors (IRFs), exerts crucial roles [4]. In addition to the central roles on the regulation of interferon or interferon stimulated genes in response to pathogen-derived danger signals [5–7], several IRF family members were also found to play critical roles in the regulation of the cell cycle and apoptosis which have important implications for understanding susceptibility to and progression of several cancers [8,9].

To date, IRF family has been currently demonstrated to include 9 members in mammals, 10 members in birds, and 11 members in fish [10]. As a crucial member of IRF family, IRF3 is a ubiquitously

* Corresponding author. Tel./fax: +86 20 89023638.
E-mail address: qinqw@scsio.ac.cn (Q. Qin).

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expressed transcription factor that controls the expression of IFN and IFN-stimulated genes (ISGs) following virus recognition [6]. Numerous innate immune receptors were capable of signaling through IRF3 to mediate their antiviral effects, including toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and DNA sensors such as DAI [5,11,12]. After virus infection, IRF3 was activated by phosphorylation at multiple serine and threonine residues in the C-terminal serine-rich region, which lead to cytoplasmic-to nuclear translocation of the phosphorylated IRF3 [13]. In addition to mammals, increased literature reported the characteristics and function of IRF3s from lower vertebrates, especially fish, including rainbow trout [14], crucian carp [15], Atlantic salmon [16], large yellow croaker [17], Japanese flounder [18] and turbot [19]. For crucian carp, fish IFN or poly-IC not only significantly up-regulated IRF3 protein expression, but also induced phosphorylation and cytoplasmic-to-nuclear translocation of IRF3. Furthermore, overexpression of crucian carp IRF3 activated IFN production that in turn triggers ISG transcription through STAT1 pathway, indicating that regulation of IFN response by vertebrate IRF3 was another ancient trait [15].

Groupers, *Epinephelus spp.* are widely cultured in China and Southeast Asian countries. However, the emergence of viral pathogens, including iridovirus and nervous necrosis virus, caused heavy economic losses in grouper aquaculture [20,21]. Singapore grouper iridovirus (SGIV), belonging to family Iridoviridae, caused more than 90% mortality in grouper and seabass [22]. The nervous necrosis virus (NNV) also caused mass mortality in grouper fry in many countries [21]. Previous studies demonstrated that grouper interferon regulatory factor 7 (Ec-IRF7) could activate zebrafish type I IFN promoter and affect SGIV replication in grouper cells [23]. However, little work has been done on other IRF members from grouper in response to virus infection. In the present study, we cloned and characterized an IRF3 homolog from grouper *Epinephelus coioides*. The potential roles of EcIRF3 in fish virus replication *in vitro* were also investigated. Our data will provide new insight into the function of fish IRF3 in responding to virus infection.

Table 1
The primers used in this study.

Primer name	Primer sequence(5′-3′)
IRF3-5′NGSP1	CATTGGGCCAGTTGTGGATCACGAGT
IRF3-5′NGSP2	TGCCAAGTCCGGGAGCCATGCTGG
IRF3-3′GSP1	GACACCCCCACGTCTAAACCATCCAG
IRF3-3′GSP2	ATGCGTTGGGTCAGATTGGCTTGGGT
IRF3-RT-F	ATGGTTTAGATGTGGGGGTGTCGGG
IRF3-RT-R	GAGGCAGAAGAACAGGGAGCACGGA
IRF3-N3-F	GAAGATCTATGTCTCATTCTAAACCACTGCTCAT
IRF3-N3-R	ACGCGTCGACTACATCTCCATCATCTCCTCGAGGG
IRF3-pET32-F	ACGCGTCGACAAATGTCTCATTCTAAACCACTGCTC
IRF3-pET32-R	CCCAAGCTTTCAGTACATCTCCATCATCTCCTCG
IRF3-pcDNA-F	GGGGTACCTATGTCTCATTCTAAACCACTGCTCAT
IRF3- pcDNA-R	GCTCTAGATCAGTACATCTCCATCATCTCCTCG
SGIV MCP-F	GCACGCTTCTCTCACCTTCA
SGIV MCP-R	AACGGCAACGGGAGCACTA
SGIV VP19-F	CTTGATGACGGAAGCGTGGT
SGIV VP19-R	TGTCAGAGGACTTGGAGAAGGAG
RGNNV CP-F	CAACTGACAACGATCACACCTTC
RGNNV CP-R	CAATCGAACACTCCAGCGACA
RGNNV RdRp-F	GTGTCCGGAGAGGTTAAGGATG
RGNNV RdRp-R	CTTGAATTGATCAACGGTGAACA
Actin-RT-F	TACGAGCTGCCTGACGGACA
Actin-RT-R	GGCTGTGATCTCCTTCTGCA
MXI-RT-F	CGAAAGTACCGTGGACGAGAA
MXI-RT-R	TGTTTGATCTGCTCCTTGACCAT
IFN-RT-F	ACTGTGTCCTTCCCGAATCATCTGT
IFN-RT-R	CAGCCTGCCTGCTTACAACACTGAGAA
ISG15-RT-F	CCTATGACATCAAAGCTGACGAGAC
ISG15-RT-R	GTGCTGTTGGCAGTGACGTTGTAGT
ISG56-RT-F	CAGGCATGGTGGAGTGGAAC
ISG56-RT-R	CTCAAGGTAGTGAACACGCGAGGTA

2. Materials and methods

2.1. Fish, cells and virus

Orange-spotted grouper, *E. coioides*, about 50 g in body weight, were purchased from a fish farm in Hainan, Guangdong province, China. Fishes were acclimatized in aerated seawater tanks at 25 °C for 7 days before use. Grouper spleen (GS) and brain (GB) cells were grown and maintained in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) at 25 °C [24,25]. Singapore grouper iridovirus (SGIV) was propagated in GS cells and red spotted grouper nervous necrosis virus (RGNNV) was propagated in GB cells. Virus stocks were stored at 80 °C until use.

2.2. Cloning sequencing of EcIRF3 and bioinformatic analysis

According to the EST sequences of EcIRF3 obtained from grouper spleen transcriptome annotation [26], primers were designed (listed in Table 1), and the 5′ and 3′ ends of the EcIRF3 cDNAs were amplified with a SMART RACE cDNA amplification kit (Clontech, USA) following the manufacturer's protocol. The corresponding PCR products were purified and then sequenced by ABI3730. After sequence assembly, the full length sequence of EcIRF3 was used for blastp analysis. The conserved domains were predicted using SMART program (http://smart.emblheidelberg.de/). Multiple amino acid sequences alignment was performed using ClustalX 1.83 and edited with GeneDoc software. The phylogenetic tree was constructed using the MEGA 4 software.

2.3. Tissue distribution pattern of EcIRF3

To determine the tissue distribution of EcIRF3, total RNA was extracted from 10 tissues from healthy orange-spotted grouper, including liver, spleen, head kidney, kidney, gill, brain, intestine, heart, skin and muscle, respectively. The expression levels of EcIRF3 in different tissues were determined by RT-PCR using EcIRF3 specific primers as described previously [27].

2.4. Construction of recombinant plasmid

The full length of open reading frame (ORF) of EcIRF3 was amplified from grouper spleen cDNA by PCR. The target PCR product was digested and sub-cloned into the prokaryotic expression vector pET-32a (Novagen, Germany) to obtain recombinant plasmid pET-EcIRF3. Similarly, the PCR product was also sub-cloned into the eukaryotic expression vector pEGFP-N3 and pcDNA3.1. All the primers used for PCR amplification were listed in Table 1. The constructed recombinant plasmids pET-EcIRF3, pcDNA-EcIRF3 and pEGFP-EcIRF3 were subsequently confirmed by DNA sequencing. To determine the effect of EcIRF3 on IFN promoter activity, zebrafish IFN1 and IFN3 promoter luciferase plasmids (IFN1/IFN3 pro-luc) were constructed as described previously [28].

2.5. Expression, purification and antiserum preparation

The expression, purification and antiserum preparation of EcIRF3 were performed as described previously [27]. Briefly, *Escherichia coli* BL21 (DE3) containing pET-EcIRF3 was induced by IPTG, and the recombinant fusion protein was purified using the HisBind purification kit (Novagen, Germany) according to the manufacture's protocol. After immunization, antiserum against

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