Interferon regulatory factor-2 in orange-spotted grouper (Epinephelus coioides): Gene, inductive expression pattern and subcellular localization

Yan Shi a,1, Zhe Zhao b,1, Jing-Kui Yin a, Xin-Ping Zhu a,c,*, Kun-Ci Chen a, De-Bo Pan a, Jian-Fang Gui d

Abstract

Interferon regulatory factor 2 (IRF-2) is a multifunctional transcription factor which exhibits both transcriptional activating and repressing activities in the IRF family. In this study, we report an IRF-2 gene isolated from orange-spotted grouper (Epinephelus coioides). The 1854 bp full-length cDNA sequence of EcIRF-2 has been cloned, encoding a putative peptide of 336 amino acids which is highly consistent with the feature of IRF family members. The genomic fragment of EcIRF-2 contains nine exons and eight introns, spanning over approximate 8.8 kb. The expression of EcIRF-2 gene was detected in various tissues of healthy orange-spotted grouper and in four tissues after being challenged with poly I:C or LPS. EcIRF-2 gene is ubiquitously expressed in various healthy fish tissues and is up-regulated in vivo in response to poly I:C or LPS. Subcellular localization analysis of EcIRF-2 suggests it is an intranuclear protein in the fish cells. We believe this research is the first report of fish IRF-2 protein localization. The results in this research establish the base for further study of function mechanism of IRF family members in orange-spotted grouper.

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1. Introduction

The immune system in fish is a complex defense system that has evolved to protect them from invading pathogenic microorganisms. A network of signaling molecules, cytokines and chemokines has been proven to control and coordinate the innate and adaptive immune responses (Liu et al., 2007). The IRF family is first and best characterized as transcriptional regulators of type I IFNs and IFN-inducible genes, and now is also recognized for a pivotal part in regulating many facets of innate and adaptive immune responses. In this family, ten members have been reported and each IRF contains a well-conserved N-terminal DNA-binding domain (DBD) of about 120 amino acids that have five conserved tryptophan repeats and bear a resemblance to the DBD of Myb transcription factors (Taniguchi et al., 2001; Veals et al., 1992). The less well-conserved C-terminal region acts as a regulatory domain and is used to classify IRFs into three groups: activators (IRF-1, IRF-3, IRF-7, IRF-9), repressors (IRF-2, IRF-8), and bi-functional ones that are able to both activate and repress gene transcription depending on the target gene (IRF-2, IRF-4, IRF-5, IRF-8) (Stellacci et al., 2004).

In mammals, IRF-2 was originally described as a transcriptional repressor by antagonizing the effect of IRF-1 (Harada et al., 1989). Further studies suggest that IRF-2 is a multifunctional transcription factor. IRF-2 exhibits both transcriptional activating and repressing activities (Shi et al., 1991). IRF-2 is a critical transacting factor that regulates in vivo responses to LPS and is a potential oncogene (Harada et al., 1993). IRF-2 also has an unexpected role in the regulation of apoptosis and cell growth (Cuesta et al., 2003). Due to the significant differences between mammals and fish, much effort has been made to study fish IRF family in recent years, and a few members have been reported, such as IRF-1, IRF-2, IRF-3 and IRF-7 (Holland et al., 2008; Kileng et al., 2009; Cai and Guo, 2008; Sun et al., 2006; Collet et al., 2003; Zhang et al., 2003).

In China, orange-spotted grouper (Epinephelus coioides) is a coral fish of high commercial value and has become a focus of considerable interest in the aquaculture. Till now, little data are available about IRFs of orange-spotted grouper when compared with other fish, such as cyprinid and salmonid fish. Therefore, extensive study on IRF family members of orange-spotted grouper would help understand its natural defense mechanism against different pathogens, especially virus which have led to frequent and heavy economic losses in the aquaculture over the past few years (Wen et al., 2008). Recently, the IRF-1 gene of orange-spotted grouper has been studied in our lab (Shi et al., 2009). As a continuing effort to better understand IRF family
members, we cloned the IRF-2 gene from orange-spotted grouper (EcIRF-2) and further characterized its features including tissue distribution, inductive expression with polyinosinic–polycytidylic acid (poly I:C) or lipopolysaccharide (LPS), and we also investigated the subcellular localization of fish IRF-2 protein. The research results in this paper provide the bases for further study of function mechanism of IRF family members in orange-spotted grouper.

2. Materials and methods

2.1. Fish and stimulation

Orange-spotted groupers (E. coioides, Perciformes, Serranidae, Epinepheline) (mass 150 g ±10 g) were obtained from Guangdong aquaculture research center at Daya Bay and maintained at 28 °C in the laboratory for one week before stimulation and sampling. Two groups of fish were injected intraperitoneally (i.p.) with poly I:C (Sigma) (500 μg of 1 mg/ml per fish) or LPS (Lipopolysaccharides from Escherichia coli 055:B5, Sigma) (500 μg of 10 μg/ml per fish) respectively, and control fish were injected with phosphate-buffered saline (PBS) (500 μl/ml per fish). The tissues of experimental groups were collected for total RNA extraction at 24, 48 and 72 h after treatment. The tissues of control fish were collected at 72 h.

2.2. RNA extraction and RACE-PCR

Total RNAs were extracted using SV Total RNA isolation System (Promega) and the first-strand cDNA was synthesized by M-MLV reverse transcriptase Kit (Promega) with random primers. RACE-PCR was performed with SMART™ RACE cDNA Amplification Kit (Clontech) according to the procedure described in the user manual. Four primers, I2-F1, I2-F2, I2-R1 and I2-R2, were used in the RACE-PCR.

2.3. DNA isolation and genomic DNA sequence amplification

Genomic DNA was isolated using standard phenol–chloroform extraction method from the liver of the orange-spotted grouper. Three pairs of primers were used to amplify genomic DNA sequence of EcIRF-2 gene, which were designed according to EcIRF-2 full-length cDNA sequence. The primers include DI2-F1 and DI2-R1, DI2-F2 and DI2-R2, DI2-F3 and DI2-R3. Cycling parameters were 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 50–55 °C for 30 s, and 72 °C for 3 min. All the PCR primers used in this study are shown in Table 1.

2.4. RT-PCR and real-time PCR

Mx and TNFα gene expressions were respectively detected by RT-PCR with primer pairs EcMx-F and EcMx-R, or EcTNFα-F and EcTNFα-R. The cycling parameters were 94 °C for 4 min, followed by 32 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. Real-time PCR was used to analyze the expression of EcIRF-2 in different conditions. Reactions were performed on ABI Prism 7300 Real-time System (Applied Biosystems) in a 20 μl volume using SYBR Green Real-time PCR Master Mix (Toyobo). Primers EcI2-F and EcI2-R were used and the cycling parameters were 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. β-actin cDNA was amplified in parallel in order to normalize the template concentrations. All samples were analyzed in triplicate, and the results were described as expression units relative to the β-actin gene in each sample using the 2^(-ΔΔC(T)) method (Livak and Schmittgen, 2001).

2.5. Plasmid construction and transfection

The plasmid used for transfection was generated by inserting the ORF of EcIRF-2 into Xho I/Eco R I sites of pEGFP-N3 vector (Clontech) with primers EcI2-F1 and EcI2-R1. The generated plasmid was verified by sequencing analysis. Transfections were carried out using Lipofectamine 2000 reagent (Invitrogen). The plasmids were transiently transfected into Epithelioma papulosum cyprini (EPC) cells, and the cells were grown in medium 199 with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin before that. For transfection, EPC cells were grown overnight to 90% confluence and then transiently transfected with pEGFP-IRF2 fusion plasmid or empty vector pEGFP-N3. The procedure was performed as described previously (Shi et al., 2008).

2.6. Subcellular localization

Transiently transfected cells were grown on glass coverslips in six-well plates. Twenty-four hours later, the transfected cells were directly observed for subcellular localization of EcIRF-2. After 48 h, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and then stained with 2.5 μg/mL Hoechst 33258 for 10 min. Green fluorescence displayed the distribution of the target protein, and the cell nucleus was indicated by the blue fluorescence of Hoechst 33258. Fluorescence signal was detected under a Leica DM IRB fluorescence microscope.

2.7. Database analysis

All sequences generated were searched for similarity using BLAST at web servers of the National Center of Biotechnology Information (NCB) (Altschul et al., 1997). Multiple alignments were performed with CLUSTALW 1.83 (Thompson et al., 1994) and GeneDoc software. Unrooted phylogenetic analysis was done using MEGA 4.0 by the Neighbor-Joining method with Poisson correction for distance calculation (Tamura et al., 2007).

3. Results

3.1. Sequence features of EcIRF-2

EcIRF-2 full-length cDNA is obtained by RACE-PCR and has 1854 bp in length with an open reading frame (ORF) of 1011 bp (GenBank accession number FJ828965), encoding 336 amino acids. The calculated molecular mass of EcIRF-2 protein is 37.73 kDa and the estimated isoelectric point (pI) is 8.39. In the 3' untranslated region of 630 bp, three mRNA instability motifs (ATTTA) are found.
Kamen, 1986). Multiple alignments indicate that, like other IRF-2 proteins, the putative EcIRF-2 protein has a DBD spanning the N-terminal 113 amino acids, including six characteristic tryptophan residues. The 160–225 amino acids of EcIRF-2 consist of the activation domain (AD). The C-terminus of EcIRF-2 is the repression domain similar to mammalian IRF-2 protein (Fig. 1) (Childs and Goodbourn, 2003). Sequence alignments show that EcIRF-2 is highly similar to snakehead fish IRF-2, with 84.5% similarity in the whole sequence. But EcIRF-2 is more similar to mandarin fish IRF-2 in the DBD sequence, with 94.6% similarity (Table 2).

Phylogenetic tree of IRF family members was built with 47 IRFs proteins from teleosts, mammals, birds and amphibians (Fig. 2). It shows that two distinct classes are retrieved, where one cluster consists of IRF-1 and IRF-2 proteins, and the other cluster contains other IRFs. In the IRF-1 and IRF-2 protein clusters, IRF-2 proteins form a distinct cluster including fish IRF-2 proteins and non-fish IRF-2 proteins.
### Table 2
Pairwise identity of EcIRF-2 with other selected IRF-2 proteins.

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**Fig. 2.** Phylogenetic relationships within the IRF family. A phylogenetic tree was constructed based on amino acid sequences of 47 IRF family members from different species including Homo sapiens (HsIRF-1: CAG46514, HsIRF-2: CAG33358, HsIRF-3: AAH17190, HsIRF-4: NM_002460, HsIRF-5: EF064718, HsIRF-6: NM_006347, HsIRF-7: AAH17190, HsIRF-8: NM_002163, HsIRF-9: NM_0060684), Rattus norvegicus (RnIRF-1: NP_036723, RnIRF-2: ABG67970, RnIRF-3: NP_00106970, RnIRF-4: NM_001106108, RnIRF-5: NM_001106586, RnIRF-6: NM_001108859, RnIRF-7: AAD4887, RnIRF-8: NM_001008722, RnIRF-9: BC079454), Mus musculus (MmIRF-1: CAJ18442, MmIRF-2: NP_032417, MmIRF-3: NP_058545, MmIRF-4: BC137713, MmIRF-5: NM_012057, MmIRF-6: NM_016851, MmIRF-8: NM_008320, MmIRF-9: NM_008394), Danio rerio (DrIRF-1: AV398364, DrIRF-2: CAX14483, DrIRF-5: EUI274624, DrIRF-6: NM_200598, DrIRF-7: AAH58298, DrIRF-8: NP_001006222, DrIRF-9: NM_205710, DrIRF-10: EU274625), Carassius auratus (CaIRF-7: AA018646, Gallus gallus (GgIRF-2: Q98925, GgIRF-10: AF380350), Epinephelus coioides (EcIRF-1: ACF95885, EcIRF-2: ACO81885/ACO81886), Ovis aries (OaIRF-2: NP_00109740), Sigmodon hispidus (ShIRF-2: Q8R4E0), Xenopus tropicalis (XrIRF-2: AAH08992), Xenopus laevis (XbIRF-2: NP_001087095), Siniperca chuatsi (ScIRF-2: AAS77257), Oncorhynchus mykiss (OmIRF-2: NP_001117910), Channa argus (ChaIRF-2: ABK63484), Salmo salar (SsIRF-2: ACI33066).
proteins. The phylogenetic tree also confirms the high similarity of EcIRF-2 with snakehead and mandarin fish IRF-2.

### 3.2. Genomic structure of EcIRF-2

The EcIRF-2 gene consists of nine exons and eight introns, spans 8834 bp covering the whole cDNA sequence (GenBank accession number FJ828966), and the locations of splice donor/acceptor sites in all introns follow the consensus ‘GT/AG’ rule (Breathnach et al., 1978). Computer searches of public genomic databases show that both IRF-2 DNA sequence of snakehead and mandarin fish IRF-2 have also been published. Snakehead fish IRF-2 has a similar structure to EcIRF-2 with nine exons and eight introns and the length of exons and introns between the two sequences is also similar (Fig. 3A). While in the mandarin fish IRF-2, there are only seven exons and six introns, and different splicing sites in the first exon and intron produce two transcripts of the same gene. Subsequent comparison between fish and mammalian IRF-2 reveals that the organization and size of exons are similar, but the mammalian IRF-2 has longer intron size than that of fish IRF-2 (Fig. 3B).

### 3.3. Expression pattern of EcIRF-2 in various tissues

EcIRF-2 transcripts were constitutively expressed in various organs of healthy orange-spotted groupers (Fig. 4A), such as gill, liver, spleen,

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**Fig. 3.** Schematic diagrams of exon–intron arrangement of IRF-2 genes from fish (A) and mammals (B). Exons and introns are indicated by the boxes and lines, respectively. The filled boxes show the coding region, while the empty ones show the untranslated region. The real lines show the introns of fish, and the broken ones show the introns of mammals. The number of nucleotides in each exon and intron is respectively shown above and below the corresponding element.

**Fig. 4.** Expression analysis of EcIRF-2 gene. (A) Tissue distribution of EcIRF-2 transcripts. Expression of EcIRF-2 in seven tissues of healthy orange-spotted groupers, including gill, liver, spleen, head kidney, brain, heart and muscle, were determined by real-time PCR. Error bars represent standard deviations obtained by measuring each sample three times from three independent experiments. (B) Expression of positive gene markers for polyIC or LPS. Three groups of orange-spotted groupers were stimulated with polyIC or LPS for 24 h, 48 h and 72 h, or with PBS (con) as control. The expression patterns of Mx or TNFα at 72 h in four tissues of treated fishes were detected by RT-PCR. (C) Expression of EcIRF-2 stimulated with polyIC or LPS. The levels of EcIRF-2 mRNA in gill, liver, spleen and head kidney of treated fishes were determined by real-time PCR. Error bars represent standard deviations obtained by measuring each tissue from three fishes. The shown data have been normalized to β-actin gene expression.
head kidney, brain, heart and muscle. The most predominant expression was seen in liver and spleen, then in muscle; while the other tissues only have low constitutive expression.

To investigate the expression pattern of EcIRF-2 transcripts upon stimulation, polyI:C and LPS were used to inject into the healthy orange-spotted groupers. Four tissues were collected and Mx or TNFα gene was used to confirm whether injection of polyI:C or LPS was effective. As shown in Fig. 4B, Mx or TNFα gene expression is up-regulated following stimulation with polyI:C or LPS. Expression pattern of EcIRF-2 was illustrated in Fig. 4C. Among the four tissues, EcIRF-2 has increased expression in three tissues, spleen, head kidney and liver. When stimulated with polyI:C, EcIRF-2 was gradually increased and peaked at 72 h in head kidney. But in spleen and liver, the increase expression was insensible. Following LPS stimulation, the expression peaked at 24 h in all the three tissues and then decreased to a low level, again rose somewhat at 72 h. In gill, the expression of EcIRF-2 decreased gradually upon polyI:C stimulation and was in a low expression level all the time with LPS stimulation.

3.4. Nuclear localization of EcIRF-2

The intracellular localization of fish IRF-2 was investigated for the first time by detecting the fluorescence distribution of EcIRF-2-GFP fusion protein in fish cells. As shown in Fig. 5, the vector-expressed
wild-type GFP was distributed in both the cytoplasm and the nucleus of fish cells, while the fusion protein EcIRF-2–GFP only accumulated in the nuclear compartment. The result shows that EcIRF-2 is an intranuclearly localized protein which is consistent with mammalian IRF-2 protein (Watanabe et al., 1991).

4. Discussion

In this study, a cDNA sequence from orange-spotted grouper, homolog of IRF-2 gene has been isolated and characterized. EcIRF-2 is believed to encode a 336 amino acid protein which contains a highly conserved N-terminal DNA-binding domain and a variable C-terminal regulatory domain. The N-terminal DBD forms a helix-turn-helix domain and recognizes a DNA element G(A)AAAG(C)T(C)GAAAG(C)T(C), termed the IRF-E (Tanaka et al., 1993). X-ray crystallography analyses reveal that each IRF-E motif is bound by two molecules of IRF-2 (Escalante et al., 1998; Fujii et al., 1999). In the C-terminus, IRF-2 has a transcriptional repression domain rich in basic residues, in contrast to IRF-1 which has a transactivation domain rich in acidic amino acids (Harada et al., 1989). In mammals, the central region between amino acids 160 and 220, is the transcriptional activation domain (AD) of IRF-2 (Yamamoto et al., 1994). The region is assumed to exist in EcIRF-2 sequence; however the drastic difference of the first twenty amino acids, especially the unique sequence “SIKHD” in EcIRF-2, needs further investigation to know its biological functions.

IRF-2 is a constitutively expressed protein in mammals (Cha and Deisseroth, 1994), and it shows constitutive expression in a wide range of tissues in vivo and in a cultured fibroblast cell line of trout (Collet et al., 2003). In this study, determination of EcIRF-2 mRNA levels reveals that the EcIRF-2 gene is expressed in various adult tissues, and significant expression is in the liver, spleen and muscle. When injected with polyl:C or LPS, the increased expression is in immune-related tissues, especially in head kidney. These results are consistent with other fish IRF-2 gene expression patterns, such as mandarin fish and snakehead fish (Sun et al., 2006; Jia and Guo, 2008). However, in the gill, the EcIRF-2 gene has a low expression level compared to those fish, and the decreased expression following stimulation of polyl:C or LPS appeared in both orange-spotted grouper and mandarin fish. Polyl:C could induce the expression of IFN and IFN-induced genes. In order to detect the effective induction of polyl:C, the positive gene marker Mx known to be up-regulated by polyl:C was used. The RT-PCR result showed that the induction was successful. The effective expression of IFN and IFN-induced genes could promote expression of IRF-2 (Cha and Deisseroth, 1994), so the increased expression of IRF-2 would keep the antiviral state in an elaborate way to prevent the host defense system from overreacting (Barnes et al., 2002a). Studies in mice with a targeted mutation in IRF-2 showed that IRF-2 knockout mice were highly refractory to LPS-induced lethality and the levels of IL-1, IL-6, IL-12, and IFN-γ mRNA or protein were significantly lower than levels seen in LPS-challenged wild-type mice (Cuesta et al., 2003). From these results, IRF-2 is believed to have a key role in LPS-induced signaling pathway.

Studies in mammals show that the IRF-2 protein is more stable and accumulates in the nucleus, which represses a number of promoters that are under the control of other IRF family members (Watanabe et al., 1991). But until now, no studies have been reported about the intracellular localization of fish IRF-2. In the study, an expression plasmid consisting of full-length EcIRF-2 and GFP was constructed to probe the localization of EcIRF-2 protein in fish cells. The subcellular localization of IRF family proteins is usually determined by the combination of nuclear localization signals (NLS) and nuclear export signals (NES) (Barnes et al., 2002b). The subcellular distribution of several IRF family proteins has been investigated, and amino acids important for their localization have been determined (Kumar et al., 2000; Lin et al., 2009). The IRF-2 proteins contain positive-acting NLS located immediately C-terminal to the DNA-binding domain, involving amino acids 120–140 in mammals, which results in the nuclear localization of IRF-2 protein (Lau et al., 2000). The similar localization of EcIRF-2 with mammalian IRF-2 protein suggests the NLS of EcIRF-2 is located in amino acids 120–140.

Acknowledgements

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**Fig. 5.** Subcellular distribution of EcIRF-2 tagged with GFP. EPC cells were transiently transfected with plasmid pEGFP-N3 or pEGFP-IRF2 for 48 h, and then examined using a fluorescence microscope. Green fluorescence shows the localization of EcIRF-2-GFP or GFP protein (left panel) and the blue images show the localization of nucleus stained by Hoechst (middle panel); Scale bars: 10 μm.
References


