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Cloning, characterization, and expression analysis of orange-spotted grouper (*Epinephelus coioides*) ILF2 gene (EcILF2)

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ABSTRACT

Interleukin-2 enhancer binding factor 2 (ILF2), also named as nuclear factor 45 (NF45), plays important roles in regulating interleukin-2 expression in mammals. In the present study, a novel ILF2 gene (designated EcILF2) was cloned and well characterized from orange-spotted grouper, *Epinephelus coioides*. The full-length EcILF2 cDNA is composed of 1544 bp and encodes a polypeptide of 387 amino acids with 98% identity to ILF2 of Atlantic salmon. The genomic DNA of EcILF2 consists of 14 exons and 13 introns, with a length of approximately 6.9 kb. EcILF2 contains two conserved domains including an RGG-rich single-stranded RNA-binding domain and a DZF zinc-finger nucleic acid binding domain. Recombinant EcILF2 was expressed in *Escherichia coli* BL21 (DE3) and purified for mouse anti-EcILF2 serum preparation. Subcellular localization analysis revealed that EcILF2 was distributed predominantly in the nucleus. Realtime quantitative PCR (RT-qPCR) analysis revealed a broad expression of EcILF2, with a relative strong expression in skin, liver, brain, head kidney and spleen. The expression of EcILF2 was differentially up-regulated after stimulation with *Vibrio vulnificus*, *Staphylococcus aureus*, *Saccharomyces cerevisiae* and Singapore grouper iridovirus (SGIV). Furthermore, EcILF2 was able to activate human IL-2 promoter in different cell lines and promote the endogenous IL-2 transcription in human H9 T cells. These results suggest that EcILF2 is potentially involved in grouper immune responses to invasion of bacterial and viral pathogens.

1. Introduction

Interleukin-2 (IL-2) is one of the most important immunomodulatory factors that plays key roles in the induction and proliferation of T cells, B cells, natural killer cells and other immune cells, like monocyte/macrophages, neutrophils and so on [1–7]. Its expression is regulated by a group of transcription factors, including AP-1, NFκB, Oct-1 and NFAT [8]. Interleukin-2 enhancer binding factor 2 (ILF2), together with interleukin-2 enhancer binding factor 3 (ILF3), were firstly identified to be the components of Jurkat T-cell NFAT complex and contribute to initiate IL-2 transcription by binding to the purine-rich antigen receptor response element 2 (ARRE-2)/NFAT target DNA sequence in the IL-2 promoter [9,10].

ILF2/ILF3 heterodimer can regulate IL-2 expression at multiple levels, such as mRNA transcription, transport, spilcing and stabilization, and translation levels [11–15]. The ILF2/ILF3 complex could interact with DNA-PK and regulate the IL-2 transcription in a cyclosporin A (CsA)-inhibitable manner, and recently, the IL-2 transactivating function of ILF2 has been demonstrated in human [12,16,17]. ILF2, together with ILF3, can regulate the IL-2 mRNA translocation from nucleus to the cytoplasm, and help the mRNA to be stabilized based on the interaction between ILF3 and IL-2 3′ UTR [18,19]. The ILF2/ILF3 heterodimer have also been identified as components of the spliceosome and were reported to participate in RNA splicing [11,20]. Furthermore, ILF2 and ILF3 could interact with the dsRNA-dependent protein kinase (PKR) [13–15] and translational initiation factor 2 [12], implying their function in regulating gene expression at translational level.

In the case of virus infection, ILF2 and ILF3 could interact specifically with some viral RNAs, including HIV TAR loop, adenovirus VA_2, and HBV RNA [21–23]. In addition, ILF2, ILF3, and RNA helicase A (RHA) associate specifically with the 5′ and 3′ non-translated regions of the bovine diarrhea virus (BVDV) genome, which is considered to be necessary for the viral translation and replication [24]. Interestingly, more recent studies have shown that

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NFAT complex also play important roles in the early and late gene expression of two other DNA virus: human polymavirus JCV and SV40 [25,26].

Recently, several ILF2 homologs have been identified in Atlantic salmon, zebrafish, grass carp, and spotted green pufferfish. However, the roles of ILF2 in IL-2 expression in bony fish have not been reported yet [27,28]. Orange-spotted grouper, *Epinephelus coioides*, the major grouper species being widely cultured in China and Southeast Asian countries, are high-priced and popular seafood fish. However, in recent years, with the rapid development of intensive marine fish farming, viral and bacterial pathogens, like Singapore grouper iridovirus (SGIV), nervous necrosis virus (VNN), and *Vibrio vulnificus*, have affected the grouper aquaculture industry causing heavy economic losses [29–31]. To better understand the immunity of *E. coioides* and investigate more immune-relevant genes, particular IL-2 associated molecules, are important for grouper disease control.

In this study, the full-length grouper EcILF2 cDNA and genomic DNA were cloned and characterized. The intracellular localization of EcILF2 in grouper embryonic (GP) cells was studied. The EcILF2 mRNA expression profiles in healthy fish tissues and pathogen-challenged fish livers were investigated by Realtime quantitative PCR (RT-qPCR). Furthermore, the transcriptional activation activity of EcILF2 on human IL-2 promoter was studied by luciferase reporter assay, and the effect of EcILF2 on endogenous IL-2 expression was analyzed in H9 T cells. To our knowledge, this is the first report demonstrating the transcriptional activation activity of fish ILF2.

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 and fed twice daily for two weeks. The fish were anesthetized using "cold-anesthetize" by dumping ice into fish bucket before killing. A series of tissue samples including head kidney, heart, liver, spleen, intestine, muscle, brain and skin were dissected from the killed fish and immediately frozen by liquid nitrogen, followed by storage at −80 °C until used.

Grouper embryonic cells (GP) were grown in Eagle's minimum essential medium containing 10% fetal bovine serum (Invitrogen, USA) at 25 °C. Human T cell line-H9 cells were maintained in RPMI 1640 supplemented with 10% FBS and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) in a humidified atmosphere containing 5% CO_2 at 37 °C.

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

V. vulnificus was originally isolated from diseased grouper, *Epinephelus awoara*. *Saccharomyces cerevisiae* and *Staphyloccocus aureus* were obtained from American Type Culture Collection (ATCC 9763 and ATCC 12598, respectively). *V. vulnificus* was cultured at 26 °C with aeration in Luria-Bertani (LB) medium prepared with fresh seawater. *S. aureus* was cultured at 37 °C in LB medium prepared with distilled water. *S. cerevisiae* was cultured in 2× YPD medium with 3% glucose (4% bacto-tryptone, 2% bacto-yeast extract (pH5.8)) at 30 °C. All 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 firstly isolated from the brown-spotted grouper, *Epinephelus tauvina*, and the propagation of SGIV was performed as described previously [30]. 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 cytopathic effect 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 tested by intraperitoneal injection (i.p). 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 pathogens used *in vivo* challenge experiments were optimized by our previous studies and were less than lethal doses. Grouper liver of five 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 1.2% agarose gel. After the total RNA had been digested with RQ1 RNase-Free DNase (Promega, USA) to remove contaminated DNA, 1 μg of total RNA was reverse transcribed to synthesize the first-strand cDNA by ReverTra Ace (TOYOBO, Japan) in a 20 μl reaction volume, containing 4 μl 5× RT buffer, 25 pmol random primers, 2 μl dNTP mixture (10 mM each) and 10 units RNase inhibitor.

2.4. Cloning and sequencing of E. coioides ILF2 (EcILF2)

2.4.1. Degenerate primers designing and initial PCR cloning

Multiple-sequence alignment of ILF2 nucleotide sequences from a variety of species were performed with the ClustalX 1.83 multiple-alignment software. The degenerate primers were designed based on the conserved nucleotides of three fish ILF2 sequences reported before, including *Danio rerio* (accession no. NM_213236), *Ctenopharyngodon idella* (accession no. DQ204681), and *Tetraodon nigroviridis* (accession no. DQ000647). The first fragment of EcILF2 gene was amplified from grouper liver cDNA by a pair of degenerate primers DF1 and DR1, using the ExTaq polymerase (Takara, Japan). PCR was conducted in Bio-Rad cycler under the following steps: initial denaturation was performed at 94 °C for 3 min, followed by 33 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, then a further 5 min extension step at 72 °C.

2.4.2. Rapid amplification of 5′ and 3′ cDNA ends (RACE) of EcILF2 cDNA

Based on the first partial sequence amplified using the degenerate primer DF1 and DR1, the 5′ and 3′ ends of EcILF2 cDNA were obtained using the RACE approaches. The first-strand cDNA templates for 5′ RACE and 3′ RACE were synthesized from the liver total RNA with the SMART™ RACE cDNA amplification kit (Clontech, USA), respectively, according the manufacturer's protocol. In detail, for the 3′ RACE, the amplification was carried out with the primer 3′ GSP2 (Table 1) and NUM (supplied by the kit). The PCR was performed at 94 °C for 3 min, followed by 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, with a final extension step of 5 min at 72 °C.

For the 5′ RACE, nested PCR method was used to get the full-length sequence of the 5′ end of EcILF2 cDNA. Briefly, the primer 5′