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Molecular cloning and characterization of FADD from the orange-spotted grouper (*Epinephelus coioides*)

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ABSTRACT

Fas-associated protein with death domain (FADD) is the key adaptor protein that transmits apoptotic signals mediated by the main death receptors. Besides being an essential instrument in cell death, FADD is also implicated in proliferation, cell cycle progression, tumor development, inflammation, innate immunity, and autophagy. In the present study, a FADD homologue (EcFADD) from the orange-spotted grouper (*Epinephelus coioides*) was cloned and its possible role in fish immunity was analyzed. The full length cDNA of EcFADD contains 808 base pairs (bp), including a 573 bp open reading frame that encodes a 190 amino acid protein with a predicted molecular mass of 21.81 kDa. Quantitative real-time polymerase chain reaction analysis indicated that EcFADD was distributed in all examined tissues. The expression of EcFADD in the spleen of *E. coioides* was differentially up-regulated when challenged with Singapore grouper iridovirus (SGIV) or polyinosine-polycytidylic acid(poly[I:C]). EcFADD was abundantly distributed in both the cytoplasm and nucleus in grouper spleen (GS) and fathead minnow (FHM) epithelial cells. Over-expression of EcFADD inhibited SGIV infection and replication and SGIV-induced apoptosis. To achieve antiviral and anti-apoptosis activities, FADD promoted the activation of interferon-stimulated response element (ISRE) and type I interferon (IFN) genes in the antiviral IFN signaling pathway and inhibited activation of apoptosis-related transcription factors p53. Our results not only characterize FADD but also reveal new immune functions and the molecular mechanisms by which FADD responds to virus infection and virus-induced apoptosis.

1. Introduction

Fas-associated death domain (FADD) is a cytosolic adaptor protein that plays a significant role in death receptor (DR)-mediated cell death [1]. Murine FADD contains two distinct functional domains with similar structure: an N-terminal death effector domain (DED) and a C-terminal death domain (DD) [2,3]. Recently, its C-terminus has been proposed as a putative third functional domain, C-terminal domain, which contains an important single phosphorylation site in a serine-rich region [4,5].

FADD is a pivotal signaling component of DR-mediated apoptosis [6]. Following DD interaction between FADD and tumor necrosis factor receptor super-family member 6 (FAS), the cytoplasmic procaspase-8 binds to FADD through DED-DED interactions [7]. This FAS-FADD-procaspase-8 complex was termed the death-inducing signaling complex (DISC) [8]. Over-expression of FADD was shown to induce apoptosis in malignant glioma cells [9] and rheumatoid synoviocytes [10]. Recently, Werner et al. reported that FADD participates in a variety of non-apoptotic processes, such as innate immune signaling, embryogenesis, hematopoiesis, lymphocyte cell cycle progression, and survival [11]. FADD is able to regulate the toll-like receptor 4 (TLR4) activation pathway through its ability to interact with myeloid differentiation primary response 88 (MyD88) [12]. Because the adaptor molecule MyD88 is necessary for response to all Toll-like receptors (TLRs) except TLR3 and a subset of TLR4 signaling events [13,14], one could expect that FADD could intervene in most TLR signaling. Absence of FADD in peripheral T lymphocytes results in inhibition of mitogen-induced T cell proliferation [2,15]. The mechanistic basis for these non-

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apoptotic activities remains unclear, but the function might depend on phosphorylation of the third functional region at the C-terminal domain [1,16]. FADD can also be involved in innate immune mechanisms independently of the TLR signaling pathway in mammalian cells [17]. Murine embryonic fibroblasts (MEF) lacking FADD were very sensitive to infection by several viruses. The antiviral effect of FADD was independent of caspase-8 and TLR3, but involved establishment of a complex named the "innateosome". This complex comprises FADD, receptor-interacting protein 1 (RIP1), and TANK-bindingkinase1 (TBK1), and leads to activation of the transcription factor interferon regulatory factor-3 (IRF-3). This signaling pathway results in the production of α and β IFNs and expression of type I IFN gene products that mediate antiviral responses. Type I IFNs then induce IRF-7 expression, which is necessary to a second cascade of IFN-α production, leading to a full antiviral response. In addition, the requirement for FADD in innate immunity against microbial pathogens may be evolutionarily conserved between insects and mammals, since a similar FADD-dependent mechanism is implicated in defense against bacteria *in Drosophila* [18].

FADD is an important pro-apoptotic adaptor in DR-induced apoptosis [19]. Apoptosis is essential for the maintenance of homeostasis in the immune system. Apoptotic cell death occurs in a wide range of viral infections [20,21]. Virus-induced apoptosis is related to activation of c-Jun N-terminal kinase, NF-κB, and p53 pathways [21,22]. Singapore grouper iridovirus (SGIV) is a major cause of mortality in fish, such as grouper and seabass [23,24]. Fish infected with SGIV have an enlarged spleen with hemorrhage and multifocal areas of splenic degeneration [23,25]. Our previous study showed that SGIV induced typical apoptosis in fathead minnow (FHM) epithelial cells [26]. Further analyses of host and/or SGIV genes based on FHM cells have contributed to our understanding of the mechanisms of iridovirus pathogenesis [27–29]. Therefore, FHM cells were used to study the function of FADD on SGIV infection in vitro.

Though many sequences of FADD have been reported in teleost, such as *Lates calcarifer* FADD, *Paralichthys olivaceus* FADD, *Salmo salar* FADD, *Oryzias latipes* FADD and so on, the function of FADD are less reported. In order to study the function of FADD in teleost, a new FADD molecule (EcFADD) from the orange-spotted grouper (*Epinephelus coioides*) was cloned and characterized in the present study. The expression profiles of EcFADD were investigated in different immune-related tissues under normal conditions and in the spleen after challenge with virus. The intercellular localization of EcFADD was detected in FHM cells. In addition, roles of EcFADD in SGIV infection were examined using various assays in both non-transfected FHM cells and in FADD-transfected FHM cells.

2. Materials and methods

2.1. Cells and viruses

Grouper spleen (GS) [30] and FHM epithelial [31] cell lines were grown in Leibovitz's L15 and M199 culture medium with 10% fetal bovine serum (Gibco, USA) at 28 °C, respectively. Propagation of SGIV was performed as described previously [32,33]. The viral titer of SGIV was 10^5 $TCID_{50}$/ml.

2.2. Fish

Juvenile orange-spotted grouper (weight 30–40 g) were purchased from Qionghai Marine Fish Farm, Hainan Province, China. Fish were maintained in a laboratory recirculating seawater system at 24–28 °C and fed twice daily for 2 weeks. Then a series of tissue samples from six fish, including kidney, heart, liver, spleen, intestine, stomach, muscle, brain, gill, head kidney, and skin, were collected and immediately frozen in liquid nitrogen followed by storage at −80 °C prior to analysis.

For the pathogen challenge tests, fish were intraperitoneally injected (i.p.) with 100 μl of polyinosine-polycytidylic acid (poly[I:C]) (1 μg/ml) purchased from Sigma-Aldrich or SGIV at a concentration of 10^5 $TCID_{50}$/ml. A group of untreated fish was used as the control. At 3, 6, 12, 24, 48, 72, 96, and 120 h post-inoculation, six fish from each group were sacrificed and the spleen was removed for quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted using the SV Total RNA Isolation System (Promega, USA) according to the manufacturer's protocol. The quality of total RNA was assessed by electrophoresis on 1% agarose gel. Total RNA was reverse transcribed to synthesize the first-strand cDNA using the ReverTra Ace kit (TOYOBO, Japan) according to the manufacturer's instructions.

2.4. Cloning and sequence analysis of EcFADD

Based on the partial sequence of FADD from *E. coioides*, the 5′ and 3′ ends of EcFADD cDNA were obtained using the rapid amplification of cDNA ends (RACE) approach. The first-strand cDNA was synthesized from the total liver RNA using the SMART™ RACE cDNA amplification kit (Clontech) for 3′ RACE and 5′ RACE. Two primers F1 (5′-GATGAA ATGGTGAGGGAGCTGCTGAAGG-3′) and R1 (5′-CTTCAGCAGCTCCCT CACCATTTCATCC-3′) were designed based on the first partial sequence. PCR was performed with 10 μM F1 or R1 and 500 nM of Nested Universal Primer A (Clontech). Denaturation was performed at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s.

PCR products were analyzed on 1% agarose gels, extracted with an AxyPrep DNA gel extraction kit (AxyGEN), ligated into pMD18-T vectors (TaKaRa), and transformed into competent *Escherichia coli* DH5 cells. Positive colonies were screened by PCR and at least two recombinant plasmids were sequenced.

2.5. Sequence analysis and amino acid alignment

Sequences were analyzed based on nucleotide and protein databases using the BLASTN and BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST/). The protein and its topology prediction were performed using software on the ExPASy Molecular Biology Server (http://expasy.pku.edu.cn). Multiple sequence alignment of the EcFADD was performed using Clustal X multiple-alignment software. The neighbor-joining (NJ) method implemented in MEGA 4.0 was used for phylogenetic analysis. The robustness of bifurcations was estimated with bootstrap analysis, and bootstrap percentages were obtained with 1000 replicates.

2.6. Real-time quantitative PCR analysis

Gene-specific primers of F2 (5′-CCTCGTCAACATTAAACGACAG GAC-3′) and R2 (5′-CAGCAGCTCCCTCACCATTTCATCC-3′) were used to verify the specificity of qRT-PCR. The β-actin gene was selected to normalize relative gene expression because of its statistically repeatable results. Two β-actin primers, actin-F (5′-GGCATCACACCTTCTACAAC GAG-3′) and actin-R (5′-AGAGGCATACAGGGACAGCACAG-3′), were used as the internal control to verify successful transcription and to calibrate the cDNA template of the corresponding samples.

qRT-PCR amplification was performed on a Roche LightCycler 480 Real-time PCR system (Roche, Switzerland) using the 2 × SYBR Green Real-time PCR Mix (TOYOBO) as previously described [34]. Relative gene expression was analyzed with the comparative Ct method ($2^{-\triangle\triangle CT}$ method). Target C_T values were normalized to the endogenous β-actin gene. Results for each treated sample were expressed as N-fold changes in target gene expression relative to the same gene target in the calibrated sample, both normalized to the β-actin gene. All