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Fish TRIM8 exerts antiviral roles through regulation of the proinflammatory factors and interferon signaling

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ABSTRACT

The tripartite motif (TRIM)-containing proteins usually exert important regulatory roles during multiple biological processes. TRIM8 has been demonstrated to be a RING domain-containing E3 ubiquitin ligase which plays critical roles in inflammation and cancer. In this study, a TRIM8 homolog from grouper, *Epinephelus coioides* (EcTRIM8) was cloned, and its effects on fish virus replication were investigated. The full-length EcTRIM8 cDNA encoded a polypeptide of 568 amino acids with 92% identity to TRIM8 homolog from large yellow croaker (*Larimichthys crocea*). Sequence alignment analysis indicated that EcTRIM8 contained conserved RING finger, B-box and coiled-coil domain. Expression patterns analysis showed that EcTRIM8 was predominant in kidney, gill, fin, liver, spleen and brain. After challenging with Singapore grouper iridovirus (SGIV) or polyinosin-polycytidylic acid (poly I:C), the EcTRIM8 transcript was significantly increased at the early stage of injection. Under fluorescence microscopy, we observed different distribution patterns of EcTRIM8 in grouper spleen (GS) cells, including punctate fluorescence evenly situated throughout the cytoplasm and bright aggregates. The ectopic expression of EcTRIM8 *in vitro* significantly inhibited the replication of SGIV and red spotted grouper nervous necrosis virus (RGNNV), evidenced by the obvious reduction in the severity of cytopathic effect (CPE) and the significant decrease in viral gene transcription and protein synthesis. Moreover, the transcription of the proinflammatory factors and interferon related immune factors were differently regulated by EcTRIM8 during SGIV or RGNNV infection. In addition, overexpression of EcTRIM8 significantly increased the transcription of interferon regulator factor 3 (IRF3) and IRF7, and enhanced IRF3 or IRF7 induced interferon-stimulated response element (ISRE) promoter activity. Together, our results firstly demonstrated that fish TRIM8 could exert antiviral function through the regulation of the expression of proinflammatory cytokines and interferon related transcription factors in response to fish viruses.

1. Introduction

The tripartite motif (TRIM) protein family was composed of multidomain ubiquitin E3 ligases characterized by the presence of three conserved domains, RING (Really Interesting New Gene), B-Box and coiled-coil (RBCC) [1]. Increased reports demonstrated that TRIMs exerted crucial roles in multiple biological processes, including cell growth, apoptosis, differentiation, carcinogenesis and antiviral immunity [2–5]. Recently, a great deal work focused great attention on the potential roles of TRIM proteins in the antiviral immune response against DNA or RNA viruses [5,6]. TRIM56 functioned as an antiviral host factor that confers resistance to yellow fever virus (YFV), dengue virus serotype 2 (DENV2), and human coronavirus (HCoV) OC43 through overlapping and distinct molecular determinants [7]. TRIM6 was found to interact with IKKε and promoted induction of IKKε-dependent IFN-stimulated genes (ISGs) [8]. In addition, TRIM25, TRIM13, and TRIM44 were also

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demonstrated to exert critical roles in antiviral immune response [5,6,9]. However, all these research efforts so far on the roles of TRIMs in antiviral immunity were mainly from the mammalian studies.

TRIM8, an important member of TRIM family, has been demonstrated to serve as a critical regulator in carcinogenesis and inflammation. *In vitro*, TRIM8 could interact with protein inhibitor of activated STAT3 (PIAS3), and the overexpression of TRIM8 abolished the negative effect of PIAS3 on signal transducer and activator of transcription 3 (STAT3), either by degradation of PIAS3 or exclusion of PIAS3 from the nucleus [10]. The ectopic expression of TRIM8 also activated NF-κB and mediated TNFα- and IL-1β-induced activation of NF-κB and K63-linked polyubiquitination of TAK1 [11]. Moreover, TRIM8 was found to physically interact with p53, impairing its interaction with murine double minute 2 (MDM2), suggested that TRIM8 could be proposed as a novel therapeutic target to enhance p53 tumor suppressor activity [12]. In addition, TRIM8 was able to interact with suppressor of cytokine signaling-1 (SOCS1), and its expression decreased the repression of interferon gamma signaling mediated by SOCS-1 [13]. Although molecular functions of mammalian TRIM8 were explored in multiple biological processes, the roles of its corresponding homolog in fish still remained largely unknown. Moreover, the potential roles of TRIM8 during virus infection also remained uncertain in both mammals and fish.

Groupers, *Epinephelus* spp. are commercially important farmed fish species in China and Southeast Asian countries. However, the outbreak of iridoviral and nodaviral diseases always caused heavy economic losses in grouper aquaculture [14–17]. As two important pathogens, Singapore grouper iridovirus (SGIV) and red spotted grouper nervous necrosis virus (RGNNV) usually caused extremely high mortality at different developmental stages of grouper [15,16]. Although several immune regulatory molecules have been illustrated to play crucial roles in grouper immune defense against fish virus infection [18–24], limited references focused on grouper TRIM family proteins [25]. Our previous reports demonstrated that grouper TRIM39 was able to inhibit grouper iridovirus and nervous necrosis virus infection *in vitro* [25]. However, no references focused on the roles of other grouper TRIMs during virus infection up to now.

In the present study, we cloned a TRIM8 homolog from marine fish and evaluated the roles of EcTRIM8 in response to fish virus infection. Our data will contribute greatly to understanding the function of TRIM genes in response to fish virus infection.

2. Materials and methods

2.1. Fish, cells and viruses

Orange-spotted groupers, *Epinephelus Coioides* (50–60 g) were purchased from a marine fish farm, Hainan Province, China. Groupers were kept in a laboratory recirculating seawater system at 25 °C for two weeks before used. Grouper spleen (GS) cells used in this study were grown in Leibovitz's L15 medium containing 10% fetal bovine serum (FBS, Gibco) at 25 °C [26]. SGIV and RGNNV were prepared and stored at −80 °C until used.

2.2. Cloning of EcTRIM8 and bioinformatic analysis

According to the sequences of several ESTs of EcTRIM8 from grouper spleen transcriptome [27], the full length cDNA of EcTRIM8 was amplified using the primers listed in Table 1. The putative open reading frame (ORF) and deduced amino acid sequences of EcTRIM8 was analyzed using BLAST program in NCBI database. The conserved domains and motifs were predicted using SMART program (http://smart.embl-heidelberg.de/). Multiple sequences alignment of TRIM8s was performed using ClustalX1.83 software. The phylogenetic tree was constructed using Mega 4.0 software.

Table 1
Primers used in this study.

Name	Sequence (5′–3′)
EcTRIM8-F	ATGGATGAAAGTTGGAAGAACTGC
EcTRIM8-R	CTTGGTCCCATAATGCTTGGTTG
EcTRIM8-Flag-F	TCAGGTACCACATGGATGAAAGTTGGAAGAACTGC
EcTRIM8-Flag-R	ACATCTCGAGCTTGGTCCCATAATGCTTGGTT
pC1-EcTRIM8-F	AATACTCGAGCTATGGCAACTTCCAGCTGTCTG
pC1-EcTRIM8-R	AATGGTACCTTAGTGTGTTGTAGGTACAGGGGTTATG
Actin- RT-F	TACGAGCTGCCTGACGGACA
Actin- RT-R	GGCTGTGATCTCCTTCTGCA
SGIV MCP- RT-F	GCACGCTTCTCTCACCTTCA
SGIV MCP- RT-R	AACGGCAACGGGAGCACTA
SGIV VP19- RT-F	TCCAAGGGAGAAACTGTAAG
SGIV VP19- RT-R	GGGGTAAGCGTGAAGACT
SGIV ICP-18-RT-F	ATCGGATCTACGTGGTTGG
SGIV ICP-18-RT-R	CCGTCGTCGGTGTCTATTC
RGNNV RdRp-RT-F	GTGTCCGGAGAGGTTAAGGATG
RGNNV RdRp-RT-R	CTTGAATTGATCAACGGTGAACA
RGNNV CP-RT-F	CAACTGACAACGATCACACCTTC
RGNNV CP-RT-R	CAATCGAACACTCCAGCGACA
EcIRF3-RT-F	ATGGTTTAGATGTGGGGGTGTCGGG
EcIRF3-RT-R	GAGGCAGAAGAACAGGGAGCACGGA
EcIRF7-RT-F	CAACACCGGATACAACCAAG
EcIRF7-RT-R	GTTCTCAACTGCTACATAGGGC
EcTNFα-RT-F	GTGTCCTGCTGTTTGCTTGGTA
EcTNFα-RT-R	CAGTGTCCGACTTGATTAGTGCTT
EcIL-1β-RT–PF	AACCTCATCATCGCCACACA
EcIL-1β-RT-PR	AGTTGCCTCACAACCGAACAC
EcIL-8-RT-PF	GCCGTCAGTGAAGGGAGTCTAG
EcIL-8-RT-PR	ATCGCAGTGGGAGTTTGCA

2.3. Expression profiles for EcTRIM8 in healthy and challenged grouper

To examine the distribution pattern of EcTRIM8 in different tissues from healthy orange-spotted grouper, including head kidney, heart, liver, spleen, intestine, muscle, brain, skin, gill, stomach and kidney, total RNA was extracted as described previously [22]. The transcripts of EcTRIM8 in different tissues were detected by quantitative real-time PCR (qRT-PCR) as described following.

To evaluate the expression profiles of EcTRIM8 in response to different stimuli, groupers were injected with PBS, SGIV, poly I:C as described previously [22]. Briefly, fish was intraperitoneal injected 100 μl individually with different stimuli, including poly I:C (1 μg/ml) and SGIV (10^5 TCID50/ml). The fish was treated with PBS as external control. Poly I:C treated groupers were collected at 0, 3, 6, 12, 24, 48 h post injection, and SGIV treated groupers were collected at 0, 3, 12, 24, 48, 72 h post infection. At indicated time points, the spleen of different groups ($n > 3$) of challenged fishes were harvested for RNA extraction and qRT-PCR analysis.

2.4. Plasmid construction

To explore the characteristic and function of EcTRIM8 *in vitro*, the full length of EcTRIM8 was amplified and cloned into pEGFP-N3 or pcDNA3.1-flag vector as described previously. All the primers were listed in Table 1, and the constructed plasmids (pEGFP-EcTRIM8 and pcDNA-EcTRIM8) were subsequently confirmed by DNA sequencing.

2.5. Cell transfection and reporter gene assay

Cell transfection was performed using Lipofectamine 2000