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Grouper TRIM13 exerts negative regulation of antiviral immune response against nodavirus

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ABSTRACT

The tripartite motif (TRIM)-containing proteins have attracted particular attention to their multiple functions in different biological processes. TRIM13, a member of the TRIM family, is a RING domain-containing E3 ubiquitin ligase which plays critical roles in diverse cellular processes including cell death, cancer and antiviral immunity. In this study, a TRIM13 homolog from orange spotted grouper, *Epinephelus coioides* (EcTRIM13) was cloned and characterized. The full-length of EcTRIM13 cDNA encoded a polypeptide of 399 amino acids which shared 81% identity with TRIM13 homolog from large yellow croaker (*Larimichthys crocea*). Amino acid alignment analysis showed that EcTRIM13 contained conserved RING finger and B-box domain. Expression patterns analysis indicated that EcTRIM13 was abundant in liver, spleen, kidney, intestine and gill. Moreover, the transcript of EcTRIM13 in grouper spleen was differently regulated after injection with Singapore grouper iridovirus (SGIV) or polyinosin-polycytidylic acid (poly I:C). Under fluorescence microscopy, we observed the tubular structure in wild type EcTRIM13 transfected cells, but the RING domain mutant resulted in the fluorescence distribution was changed and the bright punctate fluorescence was evenly situated throughout the cytoplasm, suggesting that the RING domain was essential for its accurate localization. Overexpression of EcTRIM13 *in vitro* obviously increased the replication of red spotted grouper nervous necrosis virus (RGNNV), and the enhancing effect of EcTRIM13 on virus replication was affected by the RING domain. Furthermore, the ectopic expression of EcTRIM13 not only negatively regulated the interferon promoter activity induced by interferon regulator factor (IRF) 3, IRF7, and melanoma differentiation-associated protein 5 (MDA5), but also decreased the expression of several interferon related factors. In addition, the overexpression of EcTRIM13 also differently regulated the transcription of pro-inflammatory factors. Together, our results firstly demonstrated that fish TRIM13 exerted negative regulation of antiviral response against nodavirus infection.

1. Introduction

The tripartite motif (TRIM)-containing proteins have attracted enough attention to their multiple functions in different biological processes [1,2]. Especially in antiviral immunity, a number of TRIM proteins have been elucidated to exert crucial roles in response to virus infection recently [3,4]. Knockdown of TRIM15 decreased retinoic acid-inducible gene-I (RIG-I) induced interferon production and enhanced vesicular stomatitis virus (VSV) replication [5]. Overexpression of TRIM4 potentiated virus-triggered activation of interferon regulator factor (IRF) 3 and IFN-β induction, and finally

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mildly inhibited VSV replication [6]. Moreover, several TRIM proteins were proposed to restrict human immunodeficiency virus (HIV) or murine leukemia viruses (MLV) replication upon overexpression *in vitro* [3,5,7]. In addition to the antiviral activity to RNA virus, TRIM21 and TRIM5α were also found to exert their antiviral roles against DNA viruses [8].

TRIM13/RFP2, a member of the TRIM family, has been demonstrated to play critical roles in diverse cellular processes including cell death, cancer and antiviral immunity. TRIM13 was firstly demonstrated to act as a RING E3 ubiquitin ligase and play critical roles in ER-associated degradation [9]. Further studies showed that TRIM13 regulated the translocation of caspase-8 to autophagosome and its fusion with lysosome during ER stress [10]. Furthermore, TRIM13 was able to interact with NF-kappa-B essential modulator (NEMO) and modulate its ubiquitination which may regulate IKK complex activity [11]. In addition, TRIM13 not only interacted with both melanoma differentiation-associated protein 5 (MDA5) and RIG-I *in vitro*, but also functioned as a negative regulator of MDA5-mediated type I IFN production and abolished the resistance against a lethal challenge with encephalomyocarditis virus (EMCV) [12]. Although great progresses have been made in the function of mammalian TRIM13s, no information about fish TRIM13 was available.

Groupers, Epinephelus spp. are important farmed fish species in China and Southeast Asian countries. However, the emergency of virus diseases usually caused heavy economic losses in grouper industry [13–15]. Red spotted grouper nervous necrosis virus (RGNNV) and Singapore grouper iridovirus (SGIV) infection always caused high mortality in grouper aquaculture [13,14]. Our previous studies demonstrated several grouper TRIM genes were involved in grouper iridovirus infection. Moreover, grouper TRIM39 and TRIM8 were also confirmed to act as antiviral roles against SGIV and RGNNV [16–18]. Whether grouper TRIM13 also exerted antiviral roles during grouper virus infection still remained unknown.

In the present study, a TRIM13 homolog from marine fish, orange spotted grouper (*Epinephelus coioides*), was cloned and characterized. Elucidation of the roles of TRIM13 during fish virus infection will provide new insights into the function of fish TRIMs.

2. Materials and methods

2.1. Fish, cells and viruses

Orange-spotted groupers, *E. coioides* (50–60 g) were purchased from a marine fish farm, Hainan Province, China. Groupers were kept in a laboratory recirculating seawater system before use. Grouper spleen (GS) cells used in our study were grown in Leibovitz's L15 medium containing 10% fetal bovine serum (FBS, Gibco) [19]. The stocks of SGIV and RGNNV were prepared in our lab.

2.2. Cloning of EcTRIM13, sequence analysis and plasmid construction

The full length cDNA of EcTRIM13 was amplified using the primers listed in Table 1. The putative open reading frame (ORF) and deduced amino acid sequence of EcTRIM13 was analyzed using BLAST program in NCBI database. Prediction of the functional domains and motifs was carried out using SMART program. Multiple sequences alignment of TRIM13s was performed using ClustalX1.83 software and edited using GeneDoc program. The phylogenetic tree based on the amino acid sequence was constructed using Mega 4.0 software.

To determine the function of EcTRIM13 *in vitro*, the wile type of EcTRIM13 and its RING mutant (EcTRIM13-ΔR) were subcloned into pEGFP-N3 and pcDNA3.1-flag vector as described previously. All the primers were listed in Table 1, and the recombinant plasmids (pEGFP-EcTRIM13, pEGFP-EcTRIM13-ΔR, pcDNA-EcTRIM13 and pcDNA-EcTRIM13-ΔR) were all confirmed by DNA sequencing.

Table 1
Primers used in this study.

Name	Sequence (5′–3′)
EcTRIM13-ORF-F	ATGGAGCAGCTAGAAGAGGAACT
EcTRIM13-ORF-R	CAGCTTACAGCTGCCAATAAAAC
EcTRIM13-Flag-F	TAAGGTACCGAATGGAGCAGCTAGAAGAGGAACT
EcTRIM13-Flag-R	TACTCTCGAGCAGCTTACAGCTGCCAATAAAAC
EcTRIM13-Flag-ΔR-F	TAAGGTACCGAATGCGCAAAGAGAGCCCTCACA
C1-EcTRIM13-F	TAACTCGAGCTATGGAGCAGCTAGAAGAGGAACT
C1-EcTRIM13-R	AATGGATCCCAGCTTACAGCTGCCAATAAAAC
C1-EcTRIM13-ΔR-F	TAACTCGAGCTATGCGCAAAGAGAGCCCTCACA
EcTRIM13-RT-F	TCTTGTGCGTAATTTCGATGTGA
EcTRIM13-RT-R	GCACGTAGGCTGCAAGGTTGT
Actin- RT-F	TACGAGCTGCCTGACGGACA
Actin- RT-R	GGCTGTGATCTCCTTCTGCA
SGIV MCP- RT-F	GCACGCTTCTCTCACCTTCA
SGIV MCP- RT-R	AACGGCAACGGGAGCACTA
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	GACAACAAGAACGACCCTGCTAA
EcIRF3-RT-R	GGGAGTCCGCTTGAAGATAGACA
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
EcMDA5-RT-PF	ACCTGGCTCTCAGAATTACGAACA
EcMDA5-RT-PR	TCTGCTCCTGGTGGTATTCGTTC
EcLGP2-RT-F	TGGTGGTACGCTATGGACTGC
EcLGP2-RT-R	TTGTAGCTCAGTTATCTTTGTGCGA
EcMXI-RT-F	CGAAAGTACCGTGGACGAGAA
EcMXI-RT-R	TGTTTGATCTGCTCCTTGACCAT
EcISG15-RT-F	CCTATGACATCAAAGCTGACGAGAC
EcISG15-RT-R	GTGCTGTTGGCAGTGACGTTGTAGT

2.3. Expression profiles for EcTRIM13 in grouper

To elucidate the tissue distribution pattern of EcTRIM13 in orange-spotted grouper, total RNA was extracted from 11 tissues of 3 healthy groupers, including head kidney, heart, liver, spleen, intestine, muscle, brain, skin, gill, stomach and kidney. The relative expression level of EcTRIM13 in different tissues was detected by quantitative real-time PCR (qRT-PCR) as described in the following. To illustrate the expression changes of EcTRIM13 in response to different stimuli, groupers were injected with PBS, SGIV, poly I:C as described previously [20]. Briefly, 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 injection. At different time points, the spleen of different groups ($n > 3$) of challenged grouper were collected for RNA extraction and qRT-PCR analysis.

2.4. Cell transfection and reporter gene assay

Cell transfection was performed using Lipofectamine 2000 reagent (Invitrogen) as described previously [17]. Briefly, GS cells were grown in 24-well plates, and then incubated with the mixture of Lipofectamine 2000 and different plasmids for 6 h. Then the