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TRAF3 enhances STING-mediated antiviral signaling during the innate immune activation of black carp

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ABSTRACT

Tumor necrosis factor receptor-associated factor 3 (TRAF3) is a main regulator of antiviral and anti-inflammatory pathways in mammals, which is considered to induce type I interferon (IFN) activation and negatively regulate the activation of the canonical and non-canonical NF-κB pathways. To elucidate its function in teleost fish, TRAF3 homologue of black carp (*Mylopharyngodon piceus*) has been cloned and characterized in this study. The open reading frame (ORF) of black carp TRAF3 (bcTRAF3) consists of 1722 nucleotides and bcTRAF3 contains 574 amino acids. bcTRAF3 protein migrated around 65 KDa in immunoblot analysis of both EPC and HEK293T cells. bcTRAF3 was identified as a cytosolic protein and suggested to form aggregates or be associated with vesicles scattering in the cytoplasm. It was interesting that both NF-κB and IFN transcription was activated by bcTRAF3 in reporter assay. When co-expressed with black carp STING (bcSTING), bcTRAF3 was redistributed in the cytoplasm and its subcellular location overlapped with that of bcSTING no matter what the cells was infected with GCRV or not, which suggested the association between these two molecules. bcSTING-mediated IFN production was up-regulated by bcTRAF3 in a dose dependent manner in reporter assay. Accordingly, EPC cells transfected with both bcSTING and bcTRAF3 showed enhanced antiviral activity comparing EPC cells expressing bcSTING alone. Taken together, the data generated in this paper supported the conclusion that bcTRAF3 was recruited into host innate immune activation and positively regulated bcSTING-mediated antiviral signaling.

1. Introduction

The innate immune system, an evolutionarily conserved mechanism, is the first line of host defense against pathogens, which collaborates with the adaptive immune system to help vertebrates to survive from the disadvantage environments (Jiang, 2010). The recognition of the invading microbes is crucial for the activation of host innate immune system, which relies on a limited number of germline-encoded receptors (Akira et al., 2006). These receptors have evolved to recognize the conserved products of microbial metabolism produced by microbial pathogens, but not their hosts (Janeway et al., 2002). The detection of pathogen-associated molecular patterns (PAMPs) is carried out by the pattern recognition receptors (PRRs), which include membrane-bound Toll-like receptors (TLRs) and cytosolic receptors such as RIG-I-like receptors (RLRs). PRRs activate NF-κB and interferon regulated-factor 3/7 (IRF3/7) through downstream signaling; subsequently trigger the production of pro-inflammatory cytokines, IFNs and IFN stimulated genes (ISGs), which finally initiate host innate immune response (Akira et al., 2006; Zhong et al., 2006).

Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) constitute a family of adapter proteins (TRAF1～7), which were originally discovered and characterized as signaling adaptor molecules coupled to the cytosolic regions of multiple TNF receptors (TNFRs) upon their activation. TRAF members are involved in a wide spectrum of cellular responses including cell proliferation, apoptosis, and differentiation; however, emerging evidence demonstrated that members of TRAF family played vital roles in the signaling during host immune response (Zapata et al., 2009).

Like other TRAF family members, TRAF3 exhibits a modular structure with well-defined functional domains, including a conserved TRAF domain, several Zinc finger (ZF) motifs and a RING domain (Häcker et al., 2011). Normally, TRAF3 functions as K63-specific ubiquitin ligases, which alter the function of target proteins through their non-degradative, site-specific ubiquitination activity and activate

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Table 1
Primers used in the study.

Primer name	Sequence(5′-3′)	Amplicon length (nt) and primer information
CDS		
bcTRAF3-F	ATGTCCGCAGGGCGTAATG	Full length of bcTRAF3 cDNA
bcTRAF3-R	TCAGGGGTCTGGGAGGTC	1722bp
Expression construct		
HA-bcTRAF3-F	ACTGACGGTACCATGTCCGCAGGGCGT	FRT-To-HA-bcTRAF3
HA-bcTRAF3-R	ACTGACCTCGAGTCAGGGGTCTGGGAGGT	
bcTRAF3-HA-F	ACTGACGGTACCGCCACCATGTCCGCAGGGCGT	FRT-To -bcTRAF3-HA
bcTRAF3-HA-R	ACTGACCTCGAGGGGGTCTGGGAGGTCAGA	
Flag-bcTRAF3-F	ACTGACGGTACCATGTCCGCAGGGCGT	FRT-To-Flag-bcTRAF3
Flag-bcTRAF3-R	ACTGACCTCGAGTCAGGGGTCTGGGAGGT	
q-PCR		
bc Q actin-F	TGGGCACCGCTGCTTCCT	*Ex vivo* q-PCR
bc Q actin-R	TGTCCGTCAGGCAGCTCAT	97bp
bcTRAF3-Q-F	GTCACCAGCATGACTGTCCA	*Ex vivo* q-PCR
bcTRAF3-Q-R	GGCCTCTAGTGTGGTGTTCC	157bp
bcMAVS-Q-F	ATGACAGGATCAGGGGAAT	*Ex vivo* q-PCR
bcMAVS-Q-R	ATGTTGGAAGGGGGAGTTG	192bp
bcSTING-Q-F	TGTTTGGTGCCGTTTC	*Ex vivo* q-PCR
bcSTING-Q-R	GAGCCACATTCATCTTCTTC	120bp
bcIFNa-Q-F	AAGGTGGAGGACCAGGTGAAGTTT	*Ex vivo* q-PCR
bcIFNa-Q-R	GACTCCTTATGTGATGGCTTGTGG	107bp
bcMx1-Q-F	TGAGCGTAGGCATTAGCAC	*Ex vivo* q-PCR
bcMx1-Q-R	CCTGGAGCAGCAGATAGCG	134bp
bcViperin-Q-F	CCAAAGAGCAGAAAGAGGGACC	*Ex vivo* q-PCR
bcViperin-Q-R	TCAATAGGCAAGACGAACGAGG	93bp
bcIL-1β-Q-F	ATGGAAGCGGTTGAGGTA	*Ex vivo* q-PCR
bcIL-1β-Q-R	GACACACAGGCTGGGATG	219bp
bcp52-Q-F	TATTAGATGGCAGGAAAGG	*Ex vivo* q-PCR
bcp52-Q-R	AGGTGAGTGAGTGTGAGCA	188bp
bcp50-Q-F	TCCAGGTGCGGTTTTATG	*Ex vivo* q-PCR
bcp50-Q-R	TTTGGCAGGGTCTTTTGT	198bp
bcp65-Q-F	ATACAAGCCACACCCACACG	*Ex vivo* q-PCR
bcp65-Q-R	CTTTACGCACTGAATACCCA	270bp
bcIkBα-Q-F	ACCCCTTCCTCAACATAC	*Ex vivo* q-PCR
bcIkBα-Q-R	TACCACAGTCATCCACCA	133bp
bcgig2-Q-F	AACCAGGAACCGAAGTCAG	*Ex vivo* q-PCR
bcgig2-Q-R	GCAATCCATTTTTAGAGGG	110bp

several downstream proteins (Guven-Maiorov et al., 2016). Notably, the C-terminal TRAF domain (also known as MATH domain) and the N-terminal RING domain play the critical role in TRAF3 functioning (Häcker et al., 2011; Ni et al., 2002). Previous studies demonstrated that MAVS (Mitochondrial antiviral signaling protein) (Belgnaoui et al., 2012; Paz et al., 2011; Saha et al., 2006), TRADD (Tumor necrosis factor receptor type 1-associated DEATH domain protein) (Michallet et al., 2008), CD40 (Cluster of differentiation 40) (Ni et al., 2000) and TRAF family member associated NF-κB activator (TANK) (Li et al., 2002) interacted with the MATH-TRAF3 domain, and the RING domain was proposed to act as an E3 ligase in TNF receptor signaling complexes Increasing evidence showed that TRAF3 played important roles in the signaling during host immune response, such as RLR signaling and TLR signaling (Nakhaei et al., 2008). In TLR pathway, TRAF3 associated with TRIF/IRAK1, as well as TBK1/IKKε, suggesting that TRAF3 served as a critical linker between TLR adaptors and downstream regulatory kinases important for IRF activation (Oganesyan et al., 2006). In RLR signaling, the activation of type-I IFNs is affected by the interaction between MAVS and TRAF3 (Belgnaoui et al., 2012). Except for the association of MAVS, TRAF3, a K63-linked E3 ubiquitin ligase ubiquitinating itself and downstream proteins, was found to regulate IFN but not inflammatory cytokine production during virus infection (Nakhaei et al., 2008).

Compared with its mammalian counterpart, the roles of teleost TRAF3 was under largely unknown(Chang et al., 2013; Chen et al., 2017b). TRAF3 homologues have been cloned and characterized from several species, including common carp (*Cyprinus carpio*) (Feng et al., 2011), zebrafish (*Danio rerio*), humphead snapper (*Lutjanus sanguineus*) (Cai et al., 2015) et al. In this paper, TRAF3 homologue has been cloned and characterized from black carp (Latin name: *Mylopharyngodon piceus*), which is an important freshwater industrial species and subjected to multiple pathogenic microbes such as grass carp reovirus (GCRV; double strand RNA reovirus) and spring viremia of carp virus (SVCV; single negative strand RNA rhabdovirus). This fish TRAF3 homologue activated both NF-κB and IFN in reporter assay, which was different with TRAF2 and TRAF6 of black carp (bcTRAF2 and bcTRAF6). What is more, bcTRAF3 upregulated black carp STING (Stimulator of interferon genes, also named as MITA)-mediated antiviral signaling (Lu et al., 2017), which is reported in teleost fish for the first time.

2. Materials and methods

2.1. Cells and plasmids

HEK293T, *Epithelioma papulosum cyprini* (EPC), *Ctenopharyngodon idella* kidney (CIK) and *Mylopharyngodon piceus* fin (MPF) cells were kept in the lab (Zhou et al., 2015). HEK293T cells were cultured at 37 °C with 5% CO_2; EPC, CIK and MPF cells were cultured at 25 °C with 5%