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Identification, characterization and immunological response analysis of stimulator of interferon gene (STING) from grass carp *Ctenopharyngodon idella*

Xiaoli Feng [a,1], Chunrong Yang [b,1], Yixuan Zhang [a], Limin Peng [a], Xiaohui Chen [a], Youliang Rao [a], Tianle Gu [a], Jianguo Su [a,*]

[a] College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China
[b] College of Veterinary Medicine, Northwest A&F University, Yangling 712100, China

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ABSTRACT

Stimulator of interferon gene (STING), an important adapter responsible for RLR pathway, plays a pivotal role in both viral RNA- and DNA-triggered induction of IFNs in mammals. To understand the roles of STING in piscine immune system, STING gene (CiSTING) was identified from grass carp (*Ctenopharyngodon idella*). The genomic sequence of CiSTING was of 8548 base pairs (bp), including 899 bp 5′ flank region, 7 exons and 6 introns. Promoter region was predicted and promoter activity was verified. The CiSTING cDNA was of 1358 bp with an open reading frame of 1185 bp, encoding a polypeptide of 394 amino acids with a signal peptide and three transmembrane motifs in the N-terminal region. mRNA expression of CiSTING was widespread in fifteen tissues investigated, and was up-regulated by GCRV *in vivo* and *in vitro*. Meanwhile, the transcription of CiSTING was inhibited at early stage, and then up-regulated at late phase upon poly(I:C) or PGN stimulation *in vitro*. Interestingly, CiSTING had little impact on LPS *in vitro*. In CiSTING over-expression cells, CiTBK1, CiIRF3 and CiIRF7 were significantly up-regulated post GCRV or viral/bacterial PAMPs stimulation. In addition, post GCRV or PGN stimulation, the transcription of CiIFN-I was remarkably inhibited while CiMx1 was up-regulated; as for poly(I:C) stimulation, mRNA expressions of CiIFN-I and CiMx1 were inhibited at early stage while enhanced at late phrase; after LPS stimulation, both CiIFN-I and CiMx1 were inhibited. Furthermore, antiviral activity of CiSTING was manifested by the inhibition of GCRV yield. Taken together, these results demonstrated that CiSTING may be involved in board innate immune responses via the TBK1–IRF3/IRF7 cascade, responding to not only dsRNA analogue in an IFN-dependent pathway, but also virus and bacterial PAMPs in an IFN-independent pathway. This study provided novel insights into the essential role of STING in innate immunity.

1. Introduction

As an important family of pattern-recognition receptors (PRRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) play a pivotal role in recognizing viral nucleotide pathogen-associated molecular patterns (PAMPs) in cytoplasm, triggering antiviral activity through the production of type I interferon (IFN-I) and pro-inflammatory cytokines (Kumar et al., 2011; Loo and Gale, 2011). The RLR family comprises three cytoplasmic receptors: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (Ramos and Gale, 2011; Thompson et al., 2011). Upon virus infection, RIG-I and MDA5 recognize viral nucleotides and transduce signal by the adaptor protein mitochondrial antiviral signaling (MAVS, also known as IFN-β promoter stimulator 1 (IPS-1), CARD adaptor inducing IFN-β (CARDIF), and virus-induced signaling adaptor (VISA)) that subsequently recruits and activates TANK-binding kinase 1 (TBK1) (Kawai et al., 2005; Takeuchi and Akira, 2008). Activated TBK1 phosphorylates interferon regulatory factors 3/7 (IRF3/IRF7) turning on the induction of IFN-I and IFN-stimulated genes (ISGs), such as ISG15 and myxovirus-resistant protein (Mx) (Fitzgerald et al., 2003; Sato et al., 2000).

Recently, the endoplasmic reticulum (ER)-localized protein stimulator of IFN genes (STING, also known as mediator of IRF3

* Corresponding author. Tel.: +86 29 87092139; fax: +86 29 87092164.
E-mail address: su.jianguo@gmail.com (J. Su).
[1] These authors contribute equally to this work.

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activation (MITA), transmembrane protein (TMEM173), N-terminal methionine-proline-tyrosine-serine plasma membrane tetraspanner (MPYS)) is identified as an adaptor responsible for RLR pathway but not toll-like receptor (TLR) pathway, downstream of MAVS and upstream of TBK1 and IRF3 (Ishikawa and Barber, 2008; Nakhaei et al., 2010; Zhong et al., 2008). STING is required for effective IFN-I production in response to certain RNA virus infection such as vesicular stomatitis virus (VSV), Sendai virus and dengue virus (Aguirre et al., 2012; Ishikawa and Barber, 2008). STING-knockout mice were found highly sensitive to lethal VSV infection (Ishikawa et al., 2009). STING also plays a key role in the cytosolic DNA signaling pathway that specifies IRF3 phosphorylation by TBK1 (Bowie, 2012; Tanaka and Chen, 2012). Loss of STING rendered cells fail to induce IFN-I production in response to transfection with double-stranded DNA (dsDNA) or infection with herpes simplex virus (HSV-1) or bacteria *Listeria monocytogenes* (Ishikawa et al., 2009). Therefore, STING is likely the junction adaptor molecule that integrates both RNA and DNA signaling pathways in the cytosol. In addition to the role as a signaling adaptor, recent evidence indicates that STING can associate with dsDNA directly (Abe et al., 2013; Wallach and Kovalenko, 2013), as well as cyclic dinucleotides secreted by bacteria (e.g. cyclic di-GMP, cyclic di-AMP and cyclic GAMP) to initiate IFN response (Barber, 2013; Blaauboer et al., 2014; Lam et al., 2014). Furthermore, STING is also required for a normal immune response to DNA vaccines and is crucial for adaptive immune response (Ishikawa et al., 2009).

To date, STING homologs have been identified in several fish species and functional characterizations are shown in crucian carp (*Carassius auratus* L.), zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*) (Biacchesi et al., 2012; Sun et al., 2011). Similar to mammals, fish possess a conserved RLR-triggered IFN response which is mediated by the STING-TBK1–IRF3 pathway (Biacchesi et al., 2012; Sun et al., 2011). Grass carp (*Ctenopharyngodon idella*) is a crucial aquaculture species in China and is susceptible to grass carp reovirus (GCRV, a dsRNA virus). The past several years have witnessed tremendous advances in understanding of grass carp immune system. Similar to mammals, grass carp appears to possess the functional RLR pathway, CiRIG-I, CiMDA5 and CiLGP-2 are all involved in the antiviral immune response in grass carp (Huang et al., 2010; Su et al., 2010; Yang et al., 2011). Furthermore, some pivotal signaling molecules involved in RLR signaling pathway are also identified in grass carp, including CiIPS-I (Su et al., 2011a), CiTBK1 (GenBank accession number JN704345), CiIRF3 (GenBank accession number JX999055) and CiIRF7 (GenBank accession number GQ141741). However, it is not known whether grass carp also has a homologue of mammalian STING that involved in RLR pathway. In the present study, the full-length genomic sequence of *C. idella* STING (CiSTING) was identified and characterized. The expression patterns of CiSTING were examined *in vivo* and *in vitro*. To further clarify CiSTING-mediated immune signaling pathways, the mRNA expression profiles of immune-related genes (CiTBK1, CiIRF3, CiIRF7, CiIFN-I and CiMx1) were examined post GCRV challenge or viral/bacterial PAMPs stimulation in CiSTING over-expression cells. This study aimed at functional characterizations of STING to facilitate understanding of the resistance to viral invasion and bacterial stimulation in grass carp.

2. Materials and methods

2.1. Cloning the full-length CiSTING cDNA

To identify CiSTING cDNA sequence, degenerate primers were designed according to multiple alignments of the STING sequences in *C. auratus* (GenBank accession number JF970229), *Bos taurus* (GenBank accession number BC112716), *Mus musculus* (GenBank accession number FJ222242) and *Homo sapiens* (GenBank accession number FJ222241). PCR was set up with primers MF599a and MR602a. To acquire full-length cDNA sequence of CiSTING, rapid amplification of cDNA ends (RACE) was performed using the 5′ RACE system (Invitrogen, USA) and BD SMART™ RACE cDNA amplification kit (Clontech, USA) according to the manufacturer's instructions. Forward (MF744a) and reverse (MR745a) primers were designed in the 5′ and 3′ untranslated region (UTR) to amplify the full-length coding sequence of CiSTING, respectively.

2.2. Detecting the intron(s) and identifying the 5′ flank sequence of CiSTING

Genomic DNA was extracted from grass carp spleen with the classical phenol–chlorophenol method. Five pairs of CiSTING primers, covering the full-length cDNA sequence, were employed for genomic sequence study. The sequencing results were aligned with the obtained cDNA sequence.

The 5′-flanking sequence of CiSTING gene was PCR-amplified from genomic DNA according to the protocol of Genome Walker™ Universal kit (Clontech). The first round of PCR extension was performed with primers AP1/MR973. The second round of PCR amplification was carried out with primers AP2/MR974. The PCR product was cloned into pMD18-T easy vector (TaKaRa, Japan), sequenced and overlapped with the above sequence. The full-length genomic sequence of CiSTING gene was obtained.

2.3. Sequence analysis

The searches for protein sequence similarities were conducted with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) and Matrix Global Alignment Tool (MatGAT) (http://bitincka.com/ledion/matgat/). The promoter region was predicted by WWW Promoter Scan software (http://www-bimas.cit.nih.gov/molbio/proscan/). The CpG island was predicted by online software (http://www.urogene.org/methprimer/). Simple Sequence Repeat (SSR) was searched by SSRHunter Tool (http://en.bio-soft.net/dna/SSRHunter.html). The deduced amino acid sequence was analyzed with Expert Protein Analysis System (http://www.expasy.org/). The translation was showed by Sequence Manipulation Suite (http://www.bioinformatics.org/sms/). The protein domain was predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.emblheidelberg.de/). Phylogenetic tree was constructed based on the deduced amino acid sequences by the neighbor-joining (NJ) algorithm embedded in Mega 5.1 program (Tamura et al., 2011).

2.4. Exploring the promoter activity

For detecting the promoter activity, recombinant vector pSTING-EGFP (−899/1700, containing 5′-flanking sequence, first exon (removing ATG nucleotides) and intron of CiSTING gene) was constructed. The brief procedures as follow: the DNA fragment before ATG nucleotides in the first exon was firstly amplified by premiers MF984/MR985c and inserted into *Xho*I and *Pst*I sites of pEGFP (a promoterless report vector, which was obtained from pCMV-EGFP (Clontech)). Then, second fragment containing first exon after ATG nucleotides and intron 1 was amplified by primers MF984c/MR985b and inserted into *Pst*I and *Bam*HI sites of the constructed plasmid above. Finally, the vector pSTING-EGFP was obtained and validated for sequencing.

C. idella kidney (CIK) cells (2×10^5 cells/ml) were transfected in 24-well plates with 0.5 μg of purified pSTING-EGFP and pEGFP (as a control) by FuGENE® HD Transfection Reagent (Roche, USA),