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Involvement of zebrafish RIG-I in NF-κB and IFN signaling pathways: Insights into functional conservation of RIG-I in antiviral innate immunity

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ABSTRACT

The retinoic acid-inducible gene I (RIG-I) is a critical sensor for host recognition of RNA virus infection and initiation of antiviral signaling pathways in mammals. However, data on the occurrence and functions of this molecule in lower vertebrates are limited. In this study, we characterized an RIG-I homolog (*Dr*RIG-I) from zebrafish. Structurally, this *Dr*RIG-I shares a number of conserved functional domains/motifs with its mammalian counterparts, namely, caspase activation and recruitment domain, DExD/H box, a helicase domain, and a C-terminal domain. Functionally, stimulation with *Dr*RIG-I CARD in zebrafish embryos significantly activated the NF-κB and IFN signaling pathways, leading to the expression of TNF-α, IL-8 and IFN-induced Mx, ISG15, and viperin. However, knockdown of TRIM25 (a pivotal activator for RIG-I receptors) significantly suppressed the induced activation of IFN signaling. Results suggested the functional conservation of RIG-I receptors in the NF-κB and IFN signaling pathways between teleosts and mammals, providing a perspective into the evolutionary history of RIG-I-mediated antiviral innate immunity.

1. Introduction

Host defense against viral infection depends on the recognition of specific components of invading viruses by host pattern recognition receptors (PRRs) and the subsequent production of type I IFN (IFN-I) and proinflammatory cytokines (Wu and Chen, 2014). PRRs recognize viral RNA or DNA nucleotides, including toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and the newly discovered cytosolic viral DNA sensors (Wilkins and Gale, 2010). Among these receptors, RLRs are pivotal cytosolic receptors for sensing viral RNAs. RLRs is a family of DExD/H box RNA helicases that contains RIG-I, melanoma differentiation associated factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) members (Loo and Gale, 2011; Yoneyama and Fujita, 2009). Compared with the limited knowledge for MDA5 and LGP2, RIG-I is the best characterized, no matter in the ligands or the downstream signaling pathways (Cui et al., 2008; Takahasi et al., 2008; Yoneyama et al., 2004). Structurally, RIG-I contains two N-terminal tandem caspase activation and recruitment domains (CARD), a central DExD/H box, an RNA helicase domain, and a C-terminal domain (CTD) with a repressor domain (RD) inside (Loo and Gale, 2011). CARD is the effector domain that is essential for the downstream signal transduction when RIG-I is activated. Overexpression of CARD induces constitutive signaling independent of viral infection (Yoneyama et al., 2004). The central DExD/H box has catalytic activity to unwind dsRNA in an ATP hydrolysis-dependent manner. The RD domain is responsible for the auto-inhibition of RIG-I, which can interact with CARD and the helicase domain to hinder downstream signal transduction (Saito et al., 2007). Therefore, under resting state, the expression of whole RIG-I cannot initiate immune signaling pathways (Yoneyama et al., 2004, 2005). However, after viral infection or the corresponding ligand binding, RIG-I undergoes conformational change and exposes the CARD domain to interact with downstream adaptors, therefore initiating antiviral signaling pathways, producing IFN-I and proinflammatory cytokines by activating IRF-3/-7 and NF-κB (Jiang et al., 2011; Kawai et al., 2005;

Abbreviations: RIG-I, retinoic acid-inducible gene I; PRRs, pattern recognition receptors; IFN-I, type I IFN; TLRs, toll-like receptors; RLRs, retinoic acid-inducible gene I (RIG-I)-like receptors; MDA5, melanoma differentiation associated factor 5; LGP2, laboratory of genetics and physiology 2; CARD, caspase activation and recruitment domains; CTD, C-terminal domain; RD, repressor domain; LMW, low molecular weights; ORFs, open reading frames; *Dr*Mx-pro-luc, *Danio rerio* Mx promoter luciferase; *Dr*RIG-I, *Danio rerio* RIG-I; *Dr*TRIM25, *Danio rerio* TRIM25; MO, morpholino oligonucleotides; AML, acute myeloid leukemia; HCC, hepatocellular carcinoma.

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Kowalinski et al., 2011; Saito et al., 2008). *In vitro* studies have shown that RIG-I recognizes various negative strand RNA viruses (such as Sendai virus and vesicular stomatitis virus) and several positive strand RNA viruses (such as hepatitis C virus and murine norovirus-1), but with a relatively weak recognition compared with negative strand viruses (Kato et al., 2005; McCartney et al., 2008; Meylan et al., 2005; Sumpter et al., 2005). The exact RNA structures sensed by RIG-I are believed to be the short double-stranded blunt-ended 5′-triphosphate RNAs (Schlee and Hartmann, 2010). In addition, the poly I:C with low molecular weights (LMW) was also found to be able to effectively activate RIG-I, but not MDA5 (Kohlway et al., 2013; Vissers et al., 2012).

Activation of RIG-I is believed to be precisely regulated by a number of molecular pathways. Recently, TRIM25 has been found to be essential for RIG-I activation by catalyzing the K-63 linked polyubiquitination of RIG-I at the N-terminal CARD domain (Gack et al., 2007). Typical to members of TRIM protein family, TRIM25 contains an N-terminal RING-finger E3 ligase domain, a B box/coiled-coil domain, and a C-terminal PRY/SPRY domain (Kawai and Akira, 2011). The RING-finger E3 ligase activity of TRIM25 is responsible for the delivery of polyubiquitin chain to the second CARD of RIG-I located at Lys-172 residue. Deletion of TRIM25 can significantly inhibit RIG-I signaling in mammals, leading to reduced production of IFN-I. The role of TRIM25 in RLR signaling pathway is defined to RIG-I, rather than MDA5, as there is no evidence showing that MDA5 is ubiquitinated and the ubiquitination site in RIG-I is not conserved in MDA5, although they are similar in structure and identical in downstream signaling pathway (Jiang et al., 2012).

In spite of the substantial information about RIG-I in mammals, the understanding for this molecule in lower vertebrates, including teleost fish, remains limited. Several RIG-I-like sequences have been reported in certain fish species, such as Grass carp (*Ctenopharyngodon idella*), Atlantic salmon (*Salmo salar*), Commom carp (*Cyprinus carpio*), Crucian carp (*Carassius carassius*), and Channel catfish (*Ictalurus punctatus*) (Biacchesi et al., 2009, 2012; Rajendran et al., 2012; Wan et al., 2013). However, the occurrence and functional characterization of RIG-I in fish remain elusive. In zebrafish, only a single RIG-I-like gene with incomplete sequence has been identified, which encodes a protein that is 300 aa shorter than the mammalian RIG-I proteins and lacks helicase and CTD, although the other two family members (MDA5 and LGP2) and their downstream adaptor (MAVS) are conserved between zebrafish and other species (Biacchesi et al., 2009; Lauksund et al., 2009; Xiong et al., 2012; Zou et al., 2009). Therefore, a functional RIG-I homolog is still yet to be elucidated in zebrafish.

In the present study, we identified a RIG-I (*Dr*RIG-I) from zebrafish, which contains the complete sequence of RIG-I with all the functional domains (N-terminal tandem CARD, central DExD/H box, an RNA helicase domain, and CTD with RD) in the molecule. We also demonstrated the conserved functional involvement of *Dr*RIG-I in IFN-I and NF-κB signaling pathways and the regulatory role of TRIM25 in *Dr*RIG-I participated activities. We hope that our study would enrich the current knowledge of teleost RLRs in antiviral immunity and provide valuable insights into the evolutionary history of cytosolic PRRs in innate immunity from teleost to mammals.

2. Materials and methods

2.1. Experimental fish

One-year-old male and female wild-type AB zebrafishes (*Danio rerio*) (weighing 0.5–1 g with 1–2 cm body length) were kept in tanks with recirculating water at 28 °C and fed daily with commercial pellets at 0.7% of their body weight. All fish samples were acclimatized and evaluated for overall fish health for at least 2 weeks before the experiments. Only healthy fishes, determined by their general appearance and level of activity, were used in the study.

2.2. Sequence retrieval and cloning

The N-terminal partial sequence of *Dr*RIG-I was retrieved using WU-BLAST at the Computational Biology and Functional Genomics Laboratory (http://compbio.dfci.harvard.edu/tgi/tgipage.html) and the Genewise database (http://www.ebi.ac.uk/Tools/psa/genewise/). Common carp (*Cyprinus carpio*) RIG-I amino acid sequence (ADZ55452.1) was used as the query and as a reference in the design of primers for molecular cloning of *Dr*RIG-I cDNA. The RIG-I sequences from other fish species and zebrafish TRIM25 (*Dr*TRIM25) were predicted in the Ensembl genome database (http://www.ensembl.org/index.html). Total RNA was isolated from whole fish using TRIzol reagent (Invitrogen), treated with RNase-free DNase I (QIAGEN) and reverse transcribed into first-strand cDNA using an RNA PCR kit (TaKaRa). The *Dr*RIG-I and *Dr*TRIM25 cDNAs were cloned using the primers shown in Supplemental Table S1. The 5′ and 3′-RACE core set of *Dr*RIG-I was used according to the instructions of the manufacturer (Invitrogen). PCR products were purified using a gel extraction kit (Qiagen), ligated into a pGEM-T vector (Promega) and transformed into *Escherichia coli* Top 10-competent cells (Invitrogen). Plasmid DNA was extracted by a Miniprep protocol (QIAGEN) and sequenced on a MegaBACE 1000 sequencer (GE Healthcare) using a DYEnamic ET dye terminator cycle sequencing kit (Pharmacia). Complete ORF of RIG-I was obtained by piecing together the N-terminal sequence and the C-terminal sequence obtained from 3′ RACE.

2.3. Characterization of Dr*RIG-I*

Structural characterization and conservation of *Dr*RIG-I protein were comparatively analyzed between the fishes and other species. All sequences used were retrieved from NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). Amino acid sequence identity was calculated by MEGALIGN program from DNASTAR; a multiple alignment was generated using the ClustalW program (version 1.83). Functional motifs in *Dr*RIG-I protein were analyzed using the PROSITE database (http://expasy.org/prosite/). Phylogenies of the protein sequences were estimated with MEGA5 software using parsimony and the neighbor-joining method (Kumar et al., 2008).

2.4. Plasmid constructions

The open reading frames (ORFs) of *Dr*RIG-I and *Dr*TRIM25 were inserted into pcDNA6/myc-HisB (Invitrogen) between BamHI/EcoRI and KpnI/XhoI, respectively, to construct eukaryotic expression vectors, designated as pcDNA6-*Dr*RIG-I and pcDNA6-*Dr*TRIM25. The N-terminal CARD domain of RIG-I was also inserted into pcDNA6 between BamHI/EcoRI and named pcDNA6-*Dr*RIG-I-CARD. The zebrafish Mx promoter luciferase (*Dr*Mx-pro-luc) was cloned according to previously described sequence information (Altmann et al., 2004). The NF-κB luciferase construct was purchased from Clontech (Palo Alto, CA, USA); the pRL-TK vector was obtained from Promega (Madison, USA). All primers used in plasmid construction are shown in Supplemental Table S1. All constructed sequences were confirmed by sequencing analysis. The plasmids for transfection and microinjection were prepared endotoxin-free using an EZNA™ Plasmid Midi Kit (Omega Bio-Tek, USA).

2.5. Morpholino oligonucleotide

Splice junction morpholino oligonucleotides (MO) were designed, synthesized by Gene Tools (USA), and dissolved in