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MAVS splicing variants contribute to the induction of interferon and interferon-stimulated genes mediated by RIG-I-like receptors

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ABSTRACT

The mitochondrial antiviral signaling protein (MAVS) plays a key role in the signal transduction of RIG-I-like receptors (RLRs)-mediated antiviral response. In the present study, zebrafish *MAVS transcript variants*, namely *MAVS_tv1* and *MAVS_tv2*, were cloned from zebrafish embryos. The putative MAVS_tv1 protein (full length form) contains an N-terminal CARD domain, a central proline region, and a C-terminal transmembrane domain (TM). *MAVS_tv2* is generated by a 190 bp intron fragment insertion. The putative MAVS_tv2 protein lacked TM domain due to a frame shift, with the N-terminal 303 aa residues identical to MAVS_tv1, and no sequence homology for the C-terminal 41 aa residues. Real-time PCR showed that the expression of *MAVS_tv1* in ZF4 cells was higher than that of *MAVS_tv2*, and *MAVS* variants were induced by *Edwardsiella tarda* and SVCV infection during the early time points of infection, whereas *MAVS_tv1* unchanged or *MAVS_tv2* decreased at a later time point after the infection, respectively. Overexpression of *MAVS_tv1* and *MAVS_tv2* in fish cells conferred antiviral resistance, and activated zebrafish IFN1 and IFN3 promoters. *MAVS_tv1* overexpression induced a slow (48 hpf) increased expression of *IFN1, mxa, mxb, mxe* and *RSAD2*. In contrast, *MAVS_tv2* overexpression increased rapidly and transiently the expression of *IFN1, IFN2, IFN3, mxc* and *rsad2* at 6 or 24 hpf. The simultaneous overexpression of *MAVS variants* and *RIG-I* in zebrafish embryos led to an accumulative induction of IFNs and IFN-stimulated genes including *IFN1, IFN4, mxc, mxe* and *rsad*. Furthermore, *MAVS_tv1* cooperated with *RIG-I* in the accumulation of *RIG-I* transcript in a positive feedback loop; *MAVS_tv2* synergized with *MDA5* in the accumulation of *MAVS_tv2* transcript. Collectively, these data suggest the molecular mechanisms of fish MAVS variants in antiviral immunity.

1. Introduction

Virus recognition by host pattern recognition receptors (PRRs) is essential for activation of innate antiviral immune defense and the subsequent induction of adaptive immune responses. Recognition of pathogen associated molecular patterns (PAMPs) of viruses triggers the activation of signaling pathways that result in the production of type I IFNs, IFN-stimulated genes and inflammatory cytokines, thereby suppressing viral replication (Honda et al., 2005; Melchjorsen et al., 2010). In mammals, virus-recognizing PRRs include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), HIN-200 family, and a number of intracellular DNA receptors (O'Neill and Bowie, 2010; Roberts et al., 2009; Unterholzner, 2013).

The RLRs have been known to recognize intracellular viral RNA and comprise three members, including retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (Bruns and Horvath, 2012; Yoneyama et al., 2005). RLRs bind to viral RNA through their DExD/H box RNA helicase domain and mediate the activation of TBK1 complex and NF-κB through their N-terminal caspase activation and recruitment domains (CARD) (O'Neill and Bowie, 2010; Seth et al., 2005). The adaptor protein that links RLRs to downstream signaling molecules is mitochondrial antiviral signaling protein (MAVS), also known as IFN-β promoter stimulator (IPS)-1 (Kawai et al., 2005), virus-induced signaling adaptor (VISA) (Xu et al., 2005), and CARD adaptor inducing IFN-β (Cardif) (Meylan et al., 2005). MAVS consists of an N-terminal CARD domain, a proline-rich region and a C-terminal transmembrane (TM) domain. The TM domain is responsible for targeting the protein to the mitochondrial membrane; the CARD domain of MAVS shares some homology with the first CARD domain in both MDA5 and RIG-I, and allows for the interaction with that of RLRs (Seth et al., 2005; Xu et al., 2005). The CARD

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and TM domains are both necessary and sufficient for MAVS signaling (Seth et al., 2005).

Most eukaryotic genes are alternatively spliced to produce one or more structurally related proteins. In mammals, three MAVS variants were isolated from human Hela 229 cells, and MAVS variants 1a and 1b containing uncharacterized sequences showed opposite effect on IFNβ expression, while variant 1c without the C-terminal TM had no activity on either NF-κB or IRF3 pathway (Lad et al., 2008); a miniMAVS, which was expressed from a second translational start site M142, was also identified in human cell lines, and the truncated variant without CARD domain interfered with IFN production induced by full-length MAVS (Brubaker et al., 2014). In teleost fish, MAVS orthologs have been reported from Japanese flounder (*Paralichthys olivaceus*) (Simora et al., 2010), salmon (*Salmo salar*) (Biacchesi et al., 2009; Lauksund et al., 2009), zebrafish (*Danio rerio*) (Biacchesi et al., 2009, 2012; Xiong et al., 2012), carp (*Cyprinus carpio*) (Feng et al., 2011), grass carp (*Ctenopharyngodon idella*) (Su et al., 2011; Wan et al., 2013) and spotted green pufferfish (*Tetraodon nigroviridis*) (Xiang et al., 2011). Although alternative splicing of MAVS transcripts was also found in zebrafish and salmon (Biacchesi et al., 2009), the function and mechanism of MAVS variants remains poorly understood.

In this study, we cloned two MAVS variants from zebrafish and named them as MAVS_tv1 (full-length form) and MAVS_tv2 (a truncated variant without the C-terminal TM), respectively. We demonstrated that overexpression of *MAVS_tv1* and *MAVS_tv2* in fish cells induced IFN1 and IFN3 promoters, and conferred an antiviral state against spring viremia of carp virus (SVCV). In addition, we showed the cooperative effect of *MAVS variants* and *RLRs* in inducing IFNs and IFN-stimulated genes.

2. Materials and methods

2.1. Cells and virus

ZF4 (Zebrafish embryonic fibroblast cell line) and EPC (Epithelioma papulosum cyprini) cells were maintained as described previously (Hu et al., 2014). SVCV was prepared in EPC cells at 25 °C.

2.2. Plasmids

Based on zebrafish mRNA sequences (GenBank accession Nos: FN178460 and NM_001080584), the ORFs of *MAVS_tv1* and *MAVS_tv2* were amplified with primer pairs ptGFP1-MAVS_tv1-F/ptGFP1-MAVS_tv1-R, ptGFP1-MAVS_tv2-F/ptGFP1-MAVS_tv2-R, GFPMAVS_tv1-F/GFPMAVS_tv1-R, GFPMAVS_tv2-F/GFPMAVS_tv2-R, p3×FLAG-MAVS_tv1-F/p3×FLAG-MAVS_tv1-R and p3×FLAG-MAVS_tv2-F/p3×FLAG-MAVS_tv2-R respectively, and inserted into the ptGFP1 (Chang et al., 2011), pTurboGFP-N (Everogen) and p3×FLAG-CMV-14 (Siga-Aldrich) vectors. The ORFs of zebrafish RIG-I (GenBank accession No. JX462559) and MDA5 (GenBank accession No. JX462556) were also amplified, and inserted into ptGFP1 and pcDNA3.1 (Invitrogen) vectors for constructing ptGFP1-RIG-I, ptGFP1-MDA5, pcDNA3.1-RIG-I and pcDNA3.1-MDA5. The primer sequences used for plasmid construction were described in our previous study (Zou et al., 2014) or listed in Supplementary Table S1.

2.3. Gene expression patterns modulated by bacterial and viral infection

Zebrafish ZF4 cells were passaged in six-well plates at 1×10^6 cells per well. After 24 h, these cells were infected with *Edwardsiella tarda* (PPD130/91 strain) and SVCV at a multiplicity of infection (MOI) of 1, and then collected at 6 h, 12 h and 24 h post-infection (hpi) for RNA extraction using Trizol Reagent (Gibco). For poly I:C transfection, the ZF4 cells (1×10^6 cells in 100 μl Nucleofector solution) were electroporated with 25 μg poly I:C using the Amaxa Nucleofector II transfection system (Lonza, Cologne, Germany) under Program T20. After 24 h post-transfection, the cells were collected for RNA extraction as described above. The isolated RNA was treated with RNase-free DNase I (Fermentas Life Sciences, Vilnius, Lithuania) according to the manufacturer's protocol. The first-strand cDNAs were synthesized by Superscript reverse transcriptase (Fermentas Life Sciences) and oligo(dT) primer using RNase-free DNase I-treated RNA. The length of the amplicons of *MAVS_tv1* and *MAVS_tv2* was 217 and 304 bp, respectively. Quantitative real-time PCR was performed using iQ™ SYBR Green Supermix (Bio-Rad, Singapore) on a BIO-RAD CFX96 Real-Time System under the following conditions: 3 min at 95 °C, followed by 50 cycles of 15 s at 94 °C, 15 s at 55 °C and 30 s at 72 °C. The relative expression of *MAVS variants* was normalized to the expression of *GAPDH*, and expressed as arbitrary units or fold change relative to the corresponding control group using the comparative Ct method ($2^{-\Delta\Delta Ct}$). The mean of three independent experiments was used for statistical analysis using the paired student *t* test, and $P < 0.05$ was considered to be significant. The primer sequences used for real-time PCR are described in our previous study (Zou et al., 2014) or listed in Supplementary Table S1.

2.4. Fluorescence microscopy assay

Previous studies have shown that empty vector pTurboGFP-N exhibited a global cytosolic localization in ZF4 (Zou et al., 2014) and RTG cells (Chang et al., 2011). For subcellular localization of MAVS_tv1 and MAVS_tv2, their ORFs were inserted into the pTurboGFP-N expression vector. Two microgram of pTurbo-MAVS_tv1-GFP and pTurbo-MAVS_tv2-GFP plasmids were transfected into 2×10^6 cells using Amaxa Nucleofector II transfection system (Lonza) under Program T20. Transfected ZF4 cells were stained with Hoechst 33342 (Sigma-Aldrich) before being examined under a fluorescence microscope (Zeiss, Oberkochen, Germany).

2.5. Antiviral assay

EPC cells seeded in 24-well plates at a concentration of 3×10^5 cells per well were transfected with 800 ng ptGFP1 (control), ptGFP1-MAVS_tv1 or ptGFP1-MAVS_tv2, respectively. Forty-eight hours post-transfection, transfected cells were washed and infected with SVCV at an MOI of 2, 0.2 or 0.02, respectively, and at 36 hpi, the culture supernatants were collected for the determination of virus titres by standard plaque assay. Cell monolayers were then fixed in 10% paraformaldehyde for 1 h before being stained with 0.5% crystal violet for the observation of cytopathic effect.

2.6. Luciferase activity assay

For luciferase activity assay, EPC cells seeded overnight in 24-well plates at 3×10^5 cells per well were transiently transfected with various plasmids including 200 ng zebrafish IFN1 or IFN3 promoter (Sun et al., 2011) and 20 ng pRL-TK (Promega), together with 200 ng p3×FLAG-CMV-14, MAVS_tv1-Flag or MAVS_tv2-Flag using Lipofectamine 2000 (Invitrogen). Forty-eight hours post-transfection, cells were lysed using passive lysis buffer (Promega), with luciferase activity measured on a Junior LB9509 luminometer (Berthold, Pforzheim, Germany). Data were normalized to the Renilla luciferase activity, and expressed as mean ± SD of three independent experiments.

To determine the possible association between MAVS variants and RLR variants, EPC cells seeded overnight in 24-well plates were transiently transfected with 200 ng each of MAVS variants plasmids including MAVS_tv1-Flag and MAVS_tv2-Flag, or with combinations of RLR variants plasmids including pcDNA3.1-RIG-I,