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Molecular cloning and functional characterization of duck mitochondrial antiviral-signaling protein (MAVS)

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ABSTRACT

Mitochondrial antiviral-signaling protein (MAVS), also called IPS-1/VISA/Cardif, is an important molecule involved in host defense and triggers a signal for producing type I IFN. Currently the function of MAVS in ducks (duMAVS) remains largely unclear while significant progress has been made in mammals. In this study, the full-length duMAVS cDNA was cloned from duck embryo fibroblasts (DEFs) for the first time. Tissue specificity analysis showed duMAVS was universally expressed in all detected tissues. DEFs transfected with duMAVS were able to induce interferon-β (IFN-β) expression through activating interferon regulatory factor 1 (IRF1) and nuclear factor kappa B (NF-κB). Both the CARD-like domain and transmembrane domain were required for duMAVS signaling via deletion mutant analysis. In addition, poly(I:C)- or Sendai virus (SeV)-induced IFN-β expression in DEFs were significantly decreased by knock-down of duMAVS with siRNA. Altogether, these results indicate that MAVS is a critical immunoregulator in duck innate immune system.

1. Introduction

The innate immune system is essential as the first line of defense against invasion by pathogens. The ability of hosts to recognize structurally conserved-microbial components, namely pathogen-associated molecular patterns (PAMPs), depends on the expression of host pattern recognition receptors (PRRs) (Kawai and Akira, 2010). Upon recognition, innate immune responses are initiated and type I IFN is induced, thus activating the JAK-STAT pathway to stimulate the expression of interferon-stimulated genes (ISGs), which collectively inhibit the replication of invading pathogens (Seth et al., 2005). At least three families of PRRs were recently identified, such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and RIG-I-like receptors (RLRs) (Akira et al., 2006; Meylan et al., 2006). TLRs detect PAMPs from pathogens either on the cell surface or intracellular and NLRs recognize the intracellular bacteria (Janeway Jr. and Medzhitov, 2002; Kawai and Akira, 2009). RLRs, the key cytoplasmic sensors, specifically recognize intracellular viral nucleic acid (Takeuchi and Akira, 2008; Yoneyama et al., 2004).

The adaptor protein that links RIG-I/MDA5 to the downstream signaling in mammals is the recently identified MAVS (Kawai et al., 2005), also known as IPS-1/VISA/Cardif (Meylan et al., 2006; Seth et al., 2005; Xu et al., 2005). MAVS has been reported to contain three distinct domains: an N-terminal CARD domain, a proline-rich region and a transmembrane domain. Viral RNA binding leads to the homotypic CARD—CARD interactions between RLRs and MAVS, resulting in the activation of kinase complex consisting of κB kinase (IKK)-α/β/γ and the IKK-related kinases TANK-binding kinase1 (TBK1) and IKKε. Then several transcription factors such as interferon regulatory factor-3 (IRF3) and nuclear factor-κB (NF-κB) are phosphorylated, leading to the production of type I interferons and inflammatory cytokines (McWhirter and Maniatis, 2005; Meylan et al., 2005; Wathelet et al., 1998).

Recently, there has been a growing interest in the duck immune system and several members of TLRs and RLRs have been identified in ducks (Barber et al., 2010; Cheng et al., 2015; Jia et al., 2012; Jiao et al., 2015; MacDonald et al., 2008; Wei et al., 2014; Zhai et al., 2015). Here, we firstly cloned duMAVS cDNA from duck embryo fibroblasts (DEFs) and examined the expression of duMAVS mRNA in different tissues. Furthermore, we demonstrated that duMAVS plays a crucial role in the duck IFN-β signaling pathway.

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2. Materials and methods

2.1. Cells, tissues, virus, and reagents

Duck embryo fibroblasts (DEFs) were cultured in Minima Essential Medium (MEM, Gibco, Grand Island, NY, USA) complemented with 10% fetal bovine serum (FBS, Gibco). The experimental tissues obtained from 2-month-old healthy cherry ducks, including spleen, colon, heart, oesophagus, windpipe, cerebrum, muscle, kidney, cerebellum, liver, lung, glandular stomach, duodenum, bursa of fabricius, and thymus were frozen immediately in liquid nitrogen, and stored at −80 °C until RNA extraction. Poly(I:C) was purchased from Sigma (St Louis, MO, USA) and Sendai virus (SeV) was obtained from the Center of Virus Resource and Information, Wuhan Institute of Virology, Chinese Academy of Sciences.

2.2. duMAVS cloning, sequence alignment and homology analysis

By searching the duck transcripts and genomic sequence using chicken MAVS coding sequence (Ensemble accession number ENSGALT00000025773), two duck sequences in Ensemble database with high homology to the N- and C-terminals of chicken MAVS were found. Total RNA was extracted from DEFs using Trizol reagent (Invitrogen). Briefly, a 1860 bp cDNA fragment was amplified by RT-PCR from the isolated RNA using the specific primers, duMAVS-F and duMAVS-R (Table 1). Amplicon was subcloned into the pCAGGS-Flag vector. The sequence data for duMAVS was submitted to GenBank database and assigned accession number KJ466052. Sequences were aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The phylogenic tree was generated using the MEGA 4.1 software and distances were analyzed using the neighbor-joining method. Using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/), protein domains were identified and annotated.

2.3. duMAVS tissue expression analysis

Total cellular RNA was prepared from different tissues and homogenized by using Trizol reagent (Invitrogen). Quantitative RT-PCR analysis was conducted to assess the abundance of duMAVS mRNA transcripts using SYBR Green Real-time PCR assay (Applied Biosystems, Foster City, CA, USA). The β-actin expression was used as an internal control for RNA content and integrity. All primers were designed by Primer Express software v.3.0 (Applied Biosystems) (Table 1).

2.4. Indirect immunofluorescence analysis

DEFs were trypsinized and plated onto coverslips in 24-well plates. pDsRed2-Mito (Clontech) designed for red fluorescent labeling of mitochondria was cotransfected with the duMAVS expression plasmid or an empty vector. Cells on the coverslips were fixed in 4% paraformaldehyde for 15 min after 24 h transfection. Cells were permeabilized for 10 min in a staining buffer containing Triton X-100 (0.1%) and blocked with BSA (5%) for 1 h at room temperature, then stained with anti-FLAG monoclonal Ab (MBL) followed by a secondary antibody labeled fluorescein-isothiocyanate (Invitrogen) for 1 h. Finally, the cells were treated with 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen) for 15 min. Cells imaging was carried out using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss, Zena, Germany).

Table 1
PCR primers used in the study.

Primer	Sequence(5′-3′)
duMAVS-F	GATGAATTCATGGGCTTCGCGGAGGACAAGGTGT
duMAVS-R	GACAGATCTCTATTTCTGCAGCCGGGCGTACAC
duMAVSq-F	CCGAGGCCACCGATCAC
duMAVSq-R	GGAATCCTCGTGCGTTTCTC
duMAVS(aa89-619)-F	GATGAATTCATGCAGGAGGTCTACGACCTCTAC
duMAVS(aa1-591)-R	GACAGATCTCTACCCGGGGCTGTCCCCAGGGCT
duIFNβq-F	TCTACAGAGCCTTGCCTGCAT
duIFNβq-R	TGTCGGTGTCCAAAAGGATGT
β-actin-F	GGCCAGGTCATCACCATTG
β-actin-R	GATGCCACAGGACTCCATACC

2.5. Plasmid constructions, transfection and luciferase reporter assays

Deletion mutants, duMAVS-ΔCARD (without CARD-like domain) and duMAVS-ΔTM (without transmembrane domain) expression plasmids were constructed using specific primers (Table 1). The luciferase reporter plasmids (IFN-β-Luc, 4 × NF-κB-Luc and 4 × IRF1-Luc) were constructed as previously described (Zhai et al., 2015). Empty vector and various expression plasmids with the reporter plasmid and pGL4.74 (Promega) internal control vector were transiently transfected into DEFs when approached to 80% confluence. At 24 h, cells were analyzed for luciferase expression using the dual-luciferase Assay System (Promega).

2.6. Western blotting analysis

Transiently transfected DEF cells with the expressed plasmid and mutants were collected and lysed in 150 μl 2 × lysis buffer A consisting in 65 mM Tris−HCl [pH 6.8], 4% sodium dodecyl sulfate, 3% DL-dithiothreitol and 40% glycerol. The 12% acrylamide SDS-PAGE was performed to resolve the cell lysates and then electroblotted the separated proteins onto a PVDF membrane (Millipore, Billerica, MA, USA). 10% dry milk was used to block free binding sites on membrane. Fusion proteins were analyzed by western blotting with an anti-FLAG monoclonal antibodies (MBL) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Beyotime). Signals were developed on the PVDF membrane using Super Signal West Pio Luminol kit (Pierce).

2.7. Statistical analysis

Student's t-test was used to evaluate the statistical significance of differences. P-values < 0.05 were considered statistically significant and P-values < 0.01 were considered statistically significant extremely.

3. Results and discussion

3.1. Cloning and sequence analysis of duMAVS

cDNA from DEFs RNA was used to obtain the coding region of duMAVS using the specific primers (Table 1). As shown in Fig. 1A, the full-length duMAVS was identified as a 1860 bp nucleotide cDNA sequence with an ORF encoding 619 amino acids. Sequence homology searches revealed the duMAVS had high degree of sequence similarity to multiple MAVSs in birds instead of other species. The entire amino acid sequence of duMAVS showed the highest percent identity (55.17%) to rock pigeon, followed by chicken (55.01%) and hume's groundpecker (46.62%). Moreover, duMAVS was positioned in the bird clade, exhibiting a closest evolutionary relationship with chicken MAVS (Fig. 1B).