Characterization of a MAVS ortholog from the Chinese tree shrew (Tupaia belangeri chinensis)

Ling Xu a, Dandan Yu a, Li Peng a,b, Yu Fan a,b, Jiaqi Chen c, Yong-Tang Zheng a,c, Chen Wang d, Yong-Gang Yao a,b,c,*

a Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Kunming, Yunnan 650223, China
b Kunming College of Life Science, University of Chinese Academy of Sciences, Kunming, Yunnan 650204, China
c Kunming Primate Research Center of the Chinese Academy of Sciences, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China
d State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

ABSTRACT

Human mitochondrial antiviral signaling protein (hMAVS, also known as IPS-1, VISA, or Cardif) is essential for antiviral innate immunity. The Chinese tree shrew (Tupaia belangeri chinensis), a close relative of primates, is emerging as a potential animal model for investigating viral infection. However, there is a lack of biological knowledge about the antiviral innate immunity of the tree shrew. In this study, we identified and characterized the function of the Chinese tree shrew MAVS gene (tMAVS). The cDNA of tMAVS was 2771 bp in length and encoded a polypeptide of 501 amino acids. Phylogenetic analyses based on the amino acid sequences revealed a closer affinity of tMAVS with those of primates. Quantitative real-time PCR analysis indicated that tMAVS mRNA was constitutively expressed in all seven tissues analyzed in this study. The tMAVS mRNA expression was rapidly and significantly increased after RNA virus infection. Ectopic-expression of tMAVS significantly potentiated the virus-triggered activation of IRF3, NF-κB and interferon-β (IFN-β), whereas knockdown of tMAVS displayed the opposite effect. Furthermore, tMAVS mutants lacking the caspase activation and recruitment (CARD) domains or the transmembrane (TM) domain were unable to induce IFN-β. Similar with hMAVS, mitochondrial localization of tMAVS was dependent on its domain. Collectively, this study revealed evolutionary conservation of the MAVS antiviral signaling pathway in the Chinese tree shrew.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

The tree shrew (Tupaia belangeri), a squirrel-like small mammal, has a wide distribution in Southeast Asia and Southwest China (Peng et al., 1991). Because of its unique characteristics, such as small body size, high brain-to-body mass ratio, short reproductive cycle and life span, low-cost of maintenance, and a close relationship to primates (Fan et al., 2013; Xu et al., 2013a), the tree shrew has been proposed as a viable experimental animal for making human disease models, especially for infectious diseases (Xu et al., 2013b). Previous studies have found that tree shrew is susceptible to infection with different human viruses, including hepatitis C virus (Amako et al., 2010; Xu et al., 2007), hepatitis B virus (Kock et al., 2001; Yan et al., 1984, 1996), influenza virus (Yang et al., 2013), herpes simplex virus (Rosen et al., 1985), Coxsackie virus A16 (Li et al., 2014) and EV71 (Wang et al., 2012). However, there are many lacunae, including low efficiency of infection and unknown mechanism of virus infection in this species, need to be further explored.

The innate immune system is the first line of defense against virus infection and of key importance early in viral infections. The cytoplasmic sensor of viral RNA is mainly mediated by the members of retinoic acid-inducible gene I (RIG-I) like receptor family (RLRs). The RLR family contains three members: RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation-associated gene 5) and LGP2 (laboratory of genetics and physiology 2) (Kato et al., 2006). Activation of RIG-I and MDA5 by viruses is coupled to mitochondrial antiviral signaling gene (MAVS, also known as IPS-1, Cardif or VISA) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005) through CARD:CARD domain interactions (Potter et al., 2008), which finally causes the induction of antiviral and inflammatory response mediated by IFN-β and NF-κB signaling (Belngnaoui et al., 2011). MAVS contains an N-terminal CARD domain, a
proline-rich region (PRR) in the middle, and a C-terminal trans-
membrane domain (TM). This protein was initially reported to
localize to mitochondria to exert its function (Seth et al., 2005), and
also localized to peroxisomes and mitochondrion-associated endo-
plasmic reticulum membrane (MAM) (Dixit et al., 2010; Horner et al.,
2011). Peroxisomal MAVS has been shown to be involved in the early
antiviral response, whereas mitochondrial MAVS was implicated at
a later point to promote type I interferon (IFN) and interferon-
stimulated gene (ISG) expression (Dixit et al., 2010). MAM-localized
MAVS directs innate immunity against HCV (Horner et al., 2011).
The functional differences among mitochondria-, peroxisome-, and
MAM-localized MAVS suggest that this protein recruits distinct
downstream components and set up a very powerful network in
innate immune response. Knowledge of the innate immune rec-
ognition of viral infection in tree shrew may finally help the
development of animal models for human diseases.

In this study, we identified and characterized a MAVS ortholog
from the Chinese tree shrew. We demonstrated that tMAVS has an
ability to suppress various viral replications through activation of
the IRF3/7 and NF-κB-dependent IFN responses.

2. Materials and methods

2.1. Experimental animals

The Chinese tree shrews were purchased from the experimen-
tal animal core facility of the Kunming Institute of Zoology (KIZ),
Chinese Academy of Sciences (CAS). After lethally anesthetized by
diethyl ether, we collected seven different tissues (heart, liver, spleen,
lung, kidney, intestine and brain) from 10 animals. Tissue samples
were quickly dissected and directly frozen in liquid nitrogen. All ex-
perimental procedures were performed according to the guidelines
approved by the Ethics Committee of KIZ (Approval No:
SYDW20110315001).

2.2. Total RNA extraction and reverse-transcription (RT)

Total RNA was extracted from different tissues and primary renal
cells of the tree shrew using RNAeasy Total RNA Kit (TIANGEN)
according to the manufacturer’s instructions. The A260/A280 ratio
was measured on a biophotometer (Eppendorf) and only these samples with a value of 1.8–2.0 were used for subsequent
reverse-transcription. We also evaluated the quality and integrity
of RNA samples based on the 28S and 18S rRNA bands on a 1% agarose gel. One microgram total RNA was used to synthesize cDNA
by using oligo-dT18 primer and M-MLV reverse transcriptase
(Promega).

2.3. Reverse transcription quantitative real-time PCR (RT-qPCR)

RT-qPCR was performed using the SYBR green Premix Ex Taq II
(TaKaRa, Dalian) supplemented with gene specific primers on a
MyIQ2 Two-Color Real-Time PCR Detection system (Bio-Rad, USA).
The thermal cycling protocol was 1 cycle at 95 °C for 1 min, fol-
lowed by 40 cycles of 95 °C for 15 s and 55 °C for 15 s. The resultant
PCR products were analyzed by the Bio-Rad software (Bio-Rad, USA).
In brief, a volume of 20 μL containing 0.4 μM of each upstream and
downstream primer (Table 1), 1 μL of CDNA prepared from differ-
tent tissues, and 10 μL of 2× SYBR green Premix Ex Taq II was used
for the RT-qPCR reaction. The gene expression levels were ana-
lyzed using the relative standard curve method. All quantifications
were normalized to the tree shrew β-actin and were analyzed using
the GraphPad software (GraphPad Software, La Jolla, CA, USA).

2.4. Identification and cloning of tree shrew MAVS

PCR primers for amplification of tMAVS were designed (Table 1)
based on the predicted MAVS sequences of the tree shrew re-
covered from the Ensembl (http://www.ensembl.org/index.html) and
the genome information of the Chinese tree shrew that was de-
scribed in our recent study (Fan et al., 2013) and the newly
established tree shrew database (http://www.treeeshrewdb.org) (Fan
et al., 2014). PCR amplification was performed in a volume of 20 μL
reaction mixture containing 1 μL CDNA containing 0.4 μM of each
primer, 4 mmol/L Tris–HCl (pH 8.3), 1 mmol/L MgCl₂, 20 mmol/L KCl,
80 μM of each dNTP, and one unit of LA Taq polymerase (TaKaRa).
Amplification started with a denaturation cycle at 95 °C for 1 min,
followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and ended with
a final extension cycle for 2 min at 72 °C. PCR products were puri-
fied on spin columns and directly sequenced using Big Dye
Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an
automated sequencer (ABI PRISM 3730XL, Applied Biosystems) at
the Kunming Biodiversity Large-Apparatus Regional Center, KIZ.

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Restriction endonuclease</th>
<th>Application and vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>tMAVS-qF</td>
<td>AGCATGGTCCCCCCAAGGT</td>
<td>–</td>
<td>Analytical RT-qPCR</td>
</tr>
<tr>
<td>tMAVS-qR</td>
<td>AGTTAAGGGGTCGGTCCTC</td>
<td>–</td>
<td>Analytical RT-qPCR</td>
</tr>
<tr>
<td>tIFN-β-qF</td>
<td>ACCCTAGGGAAAAACATGC</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>tIFN-β-qR</td>
<td>TTTGACTCCTGGACTATGC</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>tMAVS-F</td>
<td>ATGGTATTTGGCCGAAC</td>
<td>–</td>
<td>Fragment amplification</td>
</tr>
<tr>
<td>tMAVS-R</td>
<td>CTTGAGTGGGCCCCCGG</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>tMAVS-5′RACE</td>
<td>CTTGCGTTGGGGGCAGTCTG</td>
<td>–</td>
<td>5′ RACE</td>
</tr>
<tr>
<td>tMAVS-3′RACE-1</td>
<td>CCACTACATTGCACTGT</td>
<td>–</td>
<td>3′ RACE</td>
</tr>
<tr>
<td>tMAVS-3′RACE-2</td>
<td>AGCTGCTGCTATCCAC</td>
<td>–</td>
<td>3′ RACE nested</td>
</tr>
<tr>
<td>tMAVS-Bgl-F</td>
<td>GAGAGCTTATGATCTTCCCGGAGA</td>
<td>Bgl II</td>
<td>PCR for constructing tMAVS-EGFP using pEFGP-N2 vector</td>
</tr>
<tr>
<td>tMAVS-Kpn-R</td>
<td>GGGTTACCCTAGGTCGAGGGGCCCCCGG</td>
<td>Kpn I</td>
<td></td>
</tr>
<tr>
<td>tMAVS-ΔCARD-F</td>
<td>AGACTTATACAGTTCCTGGAGGTCCT</td>
<td>–</td>
<td>PCR for constructing mutant tMAVS without the CARD domain using tMAVS-EGFP vector</td>
</tr>
<tr>
<td>tMAVS-ΔCARD-R</td>
<td>GACATTTTCGGAGGATATAGATGTTT</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>tMAVS-STM-F</td>
<td>CTGCCTCGGCTGCGCCTTGCCGCGCCGAC</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>tMAVS-STM-R</td>
<td>GTGGCCGCTGCGGCGGCGCGCGGACAC</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>tIFN-β-pro-Kpn-F1</td>
<td>GGGTTACCTTGTTTCTTTCTTTGGTTT</td>
<td>Kpn I</td>
<td>PCR for constructing IFN-β-Luc using pGL3-Basic vector</td>
</tr>
<tr>
<td>tIFN-β-pro-Nhe-R</td>
<td>GGAAACCTGACCCCTCCCTGATGCGCATGG</td>
<td>Nhe I</td>
<td></td>
</tr>
<tr>
<td>tMAVS-BamH-F</td>
<td>CGCCGGGATCGATCTGCTGGCGGAGAACC</td>
<td>BamH I</td>
<td>PCR for constructing tMAVS-3Tag-6 using pCMV-3Tag-6 vector</td>
</tr>
<tr>
<td>tMAVS-Hind-R</td>
<td>CCCAGGCTTGGGACGGGGCGCCCAGC</td>
<td>Hind III</td>
<td></td>
</tr>
</tbody>
</table>

Restriction endonuclease sites introduced by PCR are underlined.
RT-qPCR, quantitative real-time PCR.
The complete cDNA sequence was obtained via 5’ and 3’ RACE using the SMARTer™ RACE cDNA Amplification Kit (Clontech) and 3’ Full RACE Core Set Ver.2.0 (TaKaRa), respectively. All primers were listed in Table 1. Purified PCR products were cloned into the PMD 19-T simple vector (TaKaRa). Five positive clones of each insert were sequenced.

2.5. Evolutionary analyses

To infer the phylogenetic position of the Chinese tree shrew based on the MAVS sequences, we retrieved the MAVS mRNA sequences from 11 species from GenBank: Macaca mulatta (Rhesus monkey), NM_001042666.1; Gorilla gorilla (Western gorilla), XM_004061754.1; Callithrix jacchus (white-tufted-ear marmoset), XM_002747412.2; Homo sapiens (human), NM_020746.4; Canis familiaris (dog), NM_001122609.1; Mus musculus (house mouse), NM_001206385.1; Rattus norvegicus (Norway rat), NM_001005556.1; Sus cros (pig), NM_001097429.1; Oryctolagus cuniculus (Rabbit), XM_002710888.1; Pan troglodytes (chimpanzee), XM_525410.2. Both the coding DNA sequences (CDS) and amino acid sequences were used for the phylogenetic analyses. Amino acid sequence alignment was performed using Muscle 3.7 (Edgar, 2004) with the guidance of aligned protein sequences. The dog (Canis familiaris) was used as the outgroup to root the phylogenetic tree. Trees were reconstructed according to the neighbor-joining (NJ) method and the maximum likelihood (ML) by MEGA5.0 (Tamura et al., 2011).

2.6. Plasmid construction

For epitope-tagged tMAVS constructs, PCR fragment was cloned into pEGFP-N2 with Bgl II and Kpn I and into FLAG-tagged pCMV-3Tag-6 with BamH I and Hind III. Truncated tMAVS proteins lacking the putative N-terminal CARD domain (residues 11–77) and the transmembrane (TM) domain (residues 475–497) were generated by multi-sites directed mutagenesis (Stratagene). The tree shrew IFNB1 promoter luciferase reporter (IFN-β-Luc) was constructed by using primers (Table 1) with the Kpn I and Nhe I sites that amplified region –259 to +26 of the promoter and the pGL3-basic luciferase reporter vector (Promega). All constructs were verified by sequencing.

2.7. Cell culture

HEK293 and Vero cells were grown in DMEM supplemented with 10% FBS and 1 × penicillin/streptomycin (Invitrogen, USA) at 37 °C in 5% CO2. Primary renal cells were prepared and cultured as described in our recent study (Yu et al., 2014).

2.8. Viral infection

Sendai virus (SeV) and vesicular stomatitis virus (VSV) were kindly gifts from Dr. Xinwen Chen of Wuhan Institute of Virology, CAS. Herpes simplex virus-1 (HSV-1) was introduced from Dr. Jumin Zhou’s lab at KIZ. Avian influenza virus (AIV) and Newcastle disease virus (NDV) were obtained from China Institute of Veterinary Drug Control. SeV, AIV and NDV were propagated by injecting 10-day-old specific-pathogen-free embryonated chicken egg with stocks of virus that were diluted in phosphate-buffered saline (PBS). The 50% tissue culture infective dose (TCID50) of VSV was determined by green fluorescence, and the titers of HSV-1, AIV and NDV were determined by cytopathic effect. The titers were calculated by the Reed–Muench method. The titer of SeV was determined by hemagglutination test on chicken erythrocytes.

Primary tree shrew renal cells were incubated with NDV (MOI = 10), AIV (MOI = 10), VSV (MOI = 0.01), SeV (20 HAU/mL) or HSV-1 (MOI = 10) for 1 h in DMEM without FBS, respectively, then cells were rinsed and cultured in fresh medium containing 1% FBS for the indicated times before the harvest.

2.9. Luciferase reporter assay

HEK293 cells or tree shrew renal cells were plated in 48-well plates at a density of 1 × 104 cells and cultured overnight. Cells were transfected with 0.1 μg of a Luciferase reporter vector (pGL3-tIFN-β, pNFXB-TA-Luc (Clontech), or ISRE cis-reporter (Stratagene)), together with 0.01 μg pRL-SV40-Renilla (Promega; as an internal control), the indicated amounts of an empty vector (Mock) or expression constructs using Lipofectamine™ 2000. The transfected cells were left untreated or infected with SeV (20 HAU/mL) or NDV (MOI = 10) for 12 h. Cells were lysed and detected for luciferase activity by using the Dual-Luciferase Reporter Assay System (Promega) on an infinite M1000 Pro multimode microplate reader (Tecan).

2.10. Immunofluorescence analysis

The tree shrew primary renal cells were seeded on glass coverslips and grown overnight to 40% confluence in DMEM medium supplemented with 10% FBS (Invitrogen, USA) at 37 °C in 5% CO2. Thirty-six hours after transfection with indicated vectors, cells were left untreated or infected with SeV (20 HAU/mL) for 12 h. For immunofluorescence analysis, cells were fixed with 4% paraformaldehyde for 10 min and were incubated with the rabbit anti-VISA (1:500, Sigma-Aldrich) to detect endogenous tMAVS. Mouse anti-Flag (1:1000, Abcam) was used to detect the Flag-tagged indicated proteins overnight at 4 °C. After three washes with PBS, cells were incubated with the secondary antibody (1:500, Life Technologies) for 1 h. Nuclei was stained with DAPI (Roche). Intact cells were imaged by using an Olympus FluoView™ 1000 confocal microscope (Olympus).

2.11. Western blotting and native PAGE

HEK293 cells and primary renal cells were seeded in 6-well plates at 70% confluence and were transfected with plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche). Cells were lysed on ice in RIPA lysis buffer (Beyotime). Cell lysates were centrifuged at 12,000 × g for 5 min, then were subjected to 12% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Roche) using standard procedures. The membranes were blocked with 5% nonfat dry milk in Tris buffered saline (TBS) containing 0.1% Tween 20 (TBST) at room temperature for 2 h. Then the membranes were incubated with particular primary antibody overnight at 4 °C (anti-EGFP (1:2000, Enogene) to detect tMAVS-EGFP and its mutants; rabbit anti-VISA (1:1000, Sigma-Aldrich) to detect endogenous tMAVS; mouse anti-Flag (1:5000, Abcam) to detect Flag-tagged indicated proteins; mouse anti-β-actin (1:10,000, Enogene) to detect the tree shrew and human β-actin). Membranes were washed three times with TBST for 5 min each and were incubated with anti-mouse or anti-rabbit secondary antibody (1:10,000, KPL, USA) for 1 h at room temperature. The proteins were detected using enhanced chemiluminescence (ECL) reagents (Millipore). Native PAGE was performed as previously described (Iwamura et al., 2001).

2.12. Statistical test

Comparisons between different groups were conducted by using Student’s t test (PRISM software, GraphPad Software, Inc., CA, USA). Data are presented as mean ± SEM. A P value <0.05 was considered to be significantly different.
Fig. 1. Sequence comparison and evolutionary analysis of the MAVS gene. (A) Alignment of the MAVS protein sequences of the Chinese tree shrew and other vertebrates. The CARD and the transmembrane (TM) domains were marked in the alignment. The conserved CARD region was marked in black, and the residues of the TM region as predicted by the TMpred server (http://www.ch.embnet.org/software/TMPRED_form.html) were boxed. (B) Neighbor-joining (left) and maximum likelihood (right) trees of the MAVS protein sequences of 11 vertebrate species (GenBank accession numbers are listed in Section 2). (C) Conserved synteny around the MAVS gene in the genomic sequences of the Chinese tree shrew, mouse, monkey and human (data are retrieved from the assemblies at the Ensembl website [http://www.ensembl.org] and the tree shrew genome generated in our previous study [http://www.treeshrewdb.org]).
3. Results

3.1. cDNA cloning and sequence analysis of tMAVS

According to the predicted MAVS gene sequence of the tree shrew in Ensembl and the tree shrew genome sequence generated by our own (Fan et al., 2013, 2014), we inferred that the tMAVS gene was composed of 6 exons. The full-length of tMAVS transcript had a length of 2771 bp (GenBank accession number KM005100), with a 151 bp 5′-UTR and a 1114 bp 3′-UTR (without a poly(A) tail). The open reading frame (ORF) consisted of 1506 bp and encodes 501 amino acids. Similar to human ortholog of MAVS, tMAVS contained 3 canonical functional domains: an N-terminal CARD domain, a proline rich (PRR) domain and a C-terminal TM domain. Multiple sequence alignment showed that the CARD domain of the tree shrew had the highest protein sequence identity (72%) to that of human. The TM domain was less conserved than the CARD domain and had the highest protein sequence identity (72%) to that of human ortholog (Fig. 1A).

To gain an insight into MAVS evolution, an NJ tree was constructed using the MAVS amino acid sequences of 11 representative mammalian species. We observed a clustering pattern consistent with the recognized species tree, as reported in our previous study (Fan et al., 2013). In particular, the tree shrew showed a close relationship to the primates in the NJ tree (Fig. 1B, left). We observed a similar clustering pattern when the ML approach was used (Fig. 1B, right). We further searched the conserved synteny of MAVS and other markers in the genomic region. We identified 3 markers in region where the tMAVS gene was located, and these syntenies were conserved in a variety of mammals (Fig. 1C), reinforcing the notion that tMAVS is a true ortholog.

3.2. Expression analysis of tMAVS

Constitutive mRNA expression of tMAVS was observed in the heart, liver, spleen, lung, kidney, small intestine and brain. Heart and kidney had a relatively high mRNA expression, whereas small intestine had the lowest mRNA expression (Fig. 2A). In the tree shrew primary renal cells, tMAVS expression increased upon infection of RNA virus or DNA virus. Infection with AIV, VSV, SeV, NDV or HSV-1 led to the highest level of tMAVS mRNA expression at 3 h or 6 h post-infection, followed by a decline of tMAVS mRNA level (Fig. 2B). tMAVS protein level was increased at 9 h after the infection of these viruses (Fig. 2C). There was a decrease of tMAVS upon viral infection at 3 h after the infection, although the mRNA was significantly increased at this time point. Overall, tMAVS exhibited similar expression properties to that of mammalian orthologs upon viral infections and was constitutively expressed in tree shrew primary renal cells.

3.3. tMAVS activated ISRE, NF-κB and IFN-β luciferase reporters

To determine the role of tMAVS in innate immunity, we constructed tMAVS expression vectors and explored its function in activating the ISRE, NF-κB and IFN-β luciferase reporters. As expected, ectopic-expression of tMAVS activated IFN-β-Luc reporter and potentiated the SeV-induced activation of the IFN-β-Luc in a dose-dependent manner in the tree shrew primary renal cells (Fig. 3A), which required coordinative and cooperative function of IRF3 and NF-κB (Tamura et al., 2008). Consistently, tMAVS could activate both the ISRE-Luc and NF-κB-Luc. It also potentiated the SeV-induced activation of the ISRE-Luc and NF-κB-Luc in a dose-dependent manner (Fig. 3A). Furthermore, over-expression of tMAVS in HEK293 cells markedly potentiated the SeV-induced IRF3 dimerization (Fig. 3B), which was a hallmark of IRF3 activation (Zhong et al., 2008). Since hMAVS over-expression was sufficient to delay the replication of VSV (Seth et al., 2005), we next determined whether tMAVS could mediate a similar effect in the tree shrew cells. As indicated by the diminished GFP expression, ectopic-expression of tMAVS inhibited GFP-tagged VSV replication (Fig. 3C). These results suggested that tMAVS played a critical antiviral role in the tree shrew.

3.4. tMAVS was required for virus-triggered IFN-β promoter activation

We further investigated the knockdown effect of endogenous tMAVS in virus-triggered IFN signaling. We used siRNA for tMAVS (tMAVS-siRNA) that could substantially inhibit endogenous tMAVS expression at the mRNA and protein levels (Fig. 4A). As shown in Fig. 4B, siMAVS substantially inhibited the IFN-β promoter activity and potentiated SeV-induced activation of the IFN-β-Luc. Furthermore, knockdown of tMAVS inhibited NDV- and
SeV-induced transcription of endogenous tIFNB1 gene (Fig. 4C), and significantly enhanced VSV replication (Fig. 4D). These results suggested that tMAVS was required for efficient cellular antiviral responses.

3.5. The CARD-like domain and TM domain were essential for tMAVS signaling

The N-terminal CARD-like and TM domains of MAVS were evolutionarily conserved (Fig. 1A). To determine the functions of these domains, we created expression constructs encoding MAVS mutant proteins lacking either the CARD-like domain (ΔCARD) or the TM domain (ΔTM) (Fig. 5A). As shown in Fig. 5, deletion of the CARD-like domain or the TM domain decreased the ability of MAVS to activate the ISRE, NF-κB and IFN-β promoters. Moreover, these mutants inhibited the activation of ISRE, NF-κB and IFN-β promoters by NDV. Especially, MAVS-ΔCARD completely lost its ability to activate NF-κB in response to NDV (Fig. 5C, right). Thus, the CARD-like domain and TM domain of tMAVS were essential for downstream tMAVS signaling.
tMAVS was required for virus triggered IFN-β promoter activation. (A) Effects of tMAVS-siRNA (sitMAVS) on endogenous expression of tMAVS mRNA (left) and protein (right). The tree shrew primary renal cells (1 × 10^5) were transfected with siRNA negative control (Scramble, 50 nM) or the sitMAVS (50 nM). Cells were harvested at 24 h after transfection and cell lysates were analyzed for tMAVS protein and mRNA expression. Knockdown of tMAVS caused an inhibition of virus-induced IFN-β-Luc, ISRE-Luc and NF-κB-Luc activation (B) and significant decrease of tree shrew tIFNB1 mRNA expression (C). The tree shrew primary renal cells (1 × 10^5) were transfected with the siRNA negative control (Scramble, 50 nM) or sitMAVS (50 nM) for 24 h, followed by infection with or without the indicated viruses for 12 h before the harvest. (D) Knockdown of tMAVS increased VSV replication. The tree shrew primary renal cells (1 × 10^5) were transfected with the siRNA negative control (Scramble, 50 nM) or sitMAVS (50 nM) for 12 h, followed by infected with VSV-GFP (MOI = 0.01) for 12 h. A panel of cells without any treatment was used as the control. Cells were quantified for VSV-GFP production by flow cytometry. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t test. Bars represent mean ± SEM. All experiments were repeated three times with similar results.
Fig. 5. The CARD-like domain was essential for tMAVS signaling. (A) A diagram illustrating the MAVS mutants. ΔCARD: lacking residues 11–77; ΔTM: lacking residues 475–497. Activation effect of tMAVS on the IFN-β-Luc (B), NF-κB-Luc (C) and ISRE-Luc (D) in HEK293 cells was dependent on the CARD and TM domains. Cells were co-transfected with the respective luciferase vector and the expression vector for tMAVS or its mutants for 24 h, then were left uninfected (mocked) or infected with NDV for 9 h before the harvest for luciferase assays. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \), Student’s t test. Bars represent mean ± SEM. All luciferase assays were performed three times with similar results.
3.6. Cellular localization of tMAVS

The distribution of MAVS in the mitochondria is vital for its role in signaling (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). Confocal microscopy was used to analyze the subcellular localization of the tMAVS. We found that the green fluorescent tMAVS (endogenous) was clearly merged with the red fluorescent mitochondria in primary renal cells (Fig. 6A). Exogenous tMAVS proteins were exclusively co-localized with mitochondria in the three-dimensional images (Fig. 6B, upper). However, deletion of the TM domain in tMAVS disrupted the mitochondrial localization, suggesting that mitochondrial localization was dependent on its TM domain (Fig. 6B, lower). After SeV infection, tMAVS appeared to form a dot distribution in the mitochondria (Fig. 6C), which was consistent with a previous report for a redistribution of hMAVS and the formation of densely packed, speckled MAVS puncta in virus-infected mouse embryonic fibroblasts (Xu et al., 2014).

Fig. 6. Subcellular localization of endogenous and exogenous tMAVS. (A) tMAVS was a mitochondrial protein. Endogenous tMAVS was immune-stained to show the co-localization of tMAVS with mitochondria in the tree shrew primary renal cells. (B) Localization of ectopic-expressed tMAVS to mitochondria was dependent on its TM domain. Deletion of the TM domain (ΔTM) blocks the translocation of tMAVS into mitochondria. The 3D pictures were constructed by using an Olympus FluoView™1000 confocal microscope (Olympus). (C) Formation of puncta-like tMAVS particles in the tree shrew primary renal cells infected with SeV for 12 h. For both (B) and (C), tMAVS-EGFP (the wild-type or the ΔTM mutant) and pDsRed-Mito were co-transfected (concentration ratio 10:1) into primary renal cells for 48 h, which were then imaged by confocal microscopy. The yellow staining in the overlay image indicates a co-localization of tMAVS and pDsRed-Mito. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
MAVS acts as an adaptor molecule that bridges the interactions between RIG-I/MDA5 sensing of different classes of viruses and downstream signaling (Takeuchi and Akira, 2010). The MAVS gene has been characterized in several species, including pig (Wang et al., 2008), fish (Biachesi et al., 2009; Simora et al., 2010; Su et al., 2011), chicken (Liniger et al., 2012); all these studies showed a conservation of MAVS. In our recent study of the genome of the Chinese tree shrew, a rising biomedical research model animal, we found that tree shrews lost RIG-I (DDX58) (Fan et al., 2013), which functions to recognize various RNA virus and trigger the transduction cascade involving MAVS in the signaling pathway. Under such a condition, whether the function of MAVS still conserves in the tree shrew is an open question. In this study, we aimed to characterize the MAVS homolog in tree shrew. We cloned the full-length cDNA sequence of tMAVS and performed sequence comparison analysis. Our results showed that tMAVS shared many canonical structure hallmarks with human and other mammalian MAVS proteins. tMAVS had a close affinity with that of primates (Fig. 1B), which was consistent with the recognized species tree based on whole genome information (Fan et al., 2013). Over-expression of tMAVS led to activation of the ISRE, NF-κB and IFN-β luciferase reporters, and concomitantly induced a strong antiviral ability against VSV in primary renal cells (Fig. 2). Knockdown of endogenous tMAVS showed opposite effects (Fig. 3). These observations proved that the function of tMAVS was homologous to that of other mammals.

The function of tMAVS was dependent on its TM and CARD domains, although the TM region of tMAVS exhibited relatively low protein sequence similarity (60%) compared with hMAVS (Fig. 1A). The tMAVS mutant lacking TM domain impaired its ability to activate the ISRE, NF-κB and IFN-β (Fig. 5) and mitochondrial localization (Fig. 6B). These results were consistent with the observation that the TM domain was essential for both IFN induction and MAVS localization to mitochondrial outer membrane in mammalian (Seth et al., 2005). Similarly, over-expression of the tMAVS mutant lacking the CARD domain abated its ability to activate the ISRE, NF-κB and IFN-β promoter reporters (Fig. 5), which provided direct evidence for an essential role of this domain for the tMAVS function. Taken together, our results indicated the CARD and TM domains were essential for tMAVS-mediated signaling and tMAVS was evolutionarily and functionally conserved in the Chinese tree shrew.

It is suggested that tMAVS played a role in the early stage of innate antiviral immune response (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). Upon RNA or DNA virus infection, tMAVS mRNA expression in the tree shrew primary renal cells was significantly up-regulated at 3 h after the infection and returned back to basal level at 12 h post-infection (Fig. 2B). Unexpectedly, stimulation with SeV and NDV caused a trough of the tMAVS protein expression level in early period of infection (Fig. 2). Similar tendency was also observed in previous reports (Seth et al., 2005; Xu et al., 2005). The exact cause of this pattern and its biological implications remained unknown. This pattern might reflect posttranscriptional and posttranslational regulation of RLR signaling pathway to inhibit excessive immunological and inflammatory responses (Chan and Gack, 2015; Liu et al., 2015). Further study should be carried out to clarify this important issue.

In short, we characterized a MAVS ortholog from the Chinese tree shrew and confirmed the conservation of the MAVS structure and function in this species. Further characterization of tMAVS-mediated antiviral signaling pathway in the tree shrew will be rewarding as this animal has the potential to be used for creating animal models for viral infection (Li et al., 2014; Zheng et al., 2014) and has unique genetic features in immune system (Fan et al., 2013).

Acknowledgements

We thank Dr. Xinwen Chen for providing SeV and VSV, Dr. Jumin Zhou for providing HSV-1, and Dr. Hui Zheng for providing ISRE-Luc promoter vector. We are grateful to Mr. Ronghua Luo, Mr. Yong Wu for technical assistance. This study was supported by the National Natural Science Foundation of China (U1402224), the National 863 Project of China (2012AA021801) and grants from the Chinese Academy of Sciences (KSCX2-EW-R-11).

Conflict of interests

The authors declared no conflicts of interests.

References


