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Identification and characterization of MAVS from black carp *Mylopharyngodon piceus*

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ABSTRACT

MAVS (mitochondria antiviral signaling protein) plays an important role in the host cellular innate immune response against microbial pathogens. In this study, MAVS has been cloned and characterized from black carp (*Mylopharyngodon piceus*). The full-length cDNA of black carp MAVS (bcMAVS) consists of 2352 nucleotides and the predicted bcMAVS protein contains 579 amino acids. Structural analysis showed that bcMAVS is composed of functional domains including an N-terminal CARD, a central proline-rich domain, a putative TRAF2-binding motif and a C-terminal TM domain, which is similar to mammalian MAVS. bcMAVS is constitutively transcribed in all the selected tissues including gill, kidney, heart, intestine, liver, muscle, skin and spleen; bcMAVS mRNA level in intestine, liver, muscle increased but decreased in spleen right after GCRV or SVCV infection. Multiple bands of bcMAVS were detected in western blot when it was expressed in tissue culture, which is similar to mammalian MAVS. Immunofluorescence assay determined that bcMAVS is a mitochondria protein and luciferase reporter assay demonstrated that bcMAVS could induce zebrafish IFN and EPC IFN expression in tissue culture. Data generated in this manuscript has built a solid foundation for further elucidating the function of bcMAVS in the innate immune system of black carp.

1. Introduction

Fishes and higher vertebrates enroll innate immune system and adaptive immune system to protect themselves from environmental disadvantages such as pathogen microbes invasion; however, fishes are more dependent on their innate immune system for survival from early embryonic stages of life [1–3]. The innate immune system of higher vertebrates utilizes pattern-recognition receptors (PRRs) to detect the invasion of pathogens and initiate host antiviral responses such as the induction of type I interferon and proinflammatory cytokines [4]. Three families of PRRs have clearly been shown to function in virus-specific component detection: toll-like receptors (TLRs), retinoic acidinducible gene I (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain-like receptors (NLRs) [5–7].

TLRs are a group of single, membrane-spanning, non-catalytic receptors usually expressed in sentinel cells of higher vertebrates such as macrophages and dendritic cells, which recognize structurally conserved molecules originated from microbes [5,8]. TLRs of fish have been identified and characterized from different families of several different orders, such as cyprinid, salmonid, perciform and pleuronectiform [9]. RLRs are composed of RIG-I, MDA5, and LGP2, which function as intracellular sensors of viral invasion and replication. RLRs detect viral replication through direct association with virus originated RNA, such as dsRNA from Influenza A and Sendai virus [10–12]. Extensive studies have been carried out on the RLRs of teleost fish including rainbow trout, zebrafish, grass carp and crucian carp [13,14]. NLRs are intracellular sensors of pathogen-associated molecular patterns (PAMPs) that enter the cell through phagocytosis or pores and damage-associated molecular patterns (DAMPs) that are associated with cell stress. NLRs can cooperate with TLRs and regulate inflammatory and apoptotic response [7]. Multiple NLR gene orthologs in fish have been reported and fish NLRs are considered to form a large multigene family in teleostei [15].

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RLRs are known to play an important role in sensing RNA virus invasion in the higher vertebrates. RIG-I and MDA5 trigger the host immune response upon recognition of viral dsRNA through the N-terminal caspase recruitment domain (CARD). Because of the absence of CARD domains, LGP2 could not induce the immune signaling alone, however, LGP2 is necessary for the effective host cellular immune response against virus initiated by RIG-I and MDA5 [16,17]. MAVS (mitochondria antiviral signaling protein), also known as IPS-1/VISA/Cardif, is the downstream adaptor in RIG-I and MDA5 signaling that trigger host innate immune response against virus infection [18–21]. MAVS of higher vertebrates is usually composed of an N-terminal CARD domain, a central proline-rich region and a C-terminal transmembrane (TM) domain. MAVS is located on the outer membrane of mitochondria through its TM domain and its CARD domain interacts with the CARD domain of RIG-I and MDA5 when virus derived RNA is recognized by these two sensors [22,23]. MAVS is activated upon association with RIG-I and MDA5 and result in activation of IRF-3/7 and NF-κB, which lead to the induction of type I IFNs and IFN-stimulated genes (ISGs) [24,25].

Extensive studies on fish immune system have been reported because of the intensive farming of different commercial fish species and the acknowledgment of zebrafish as a powerful new vertebrate model of biomedical research [26,27]. MAVS cDNA of several fishes have been cloned and characterized, including Atlantic salmon, zebrafish, fathead minnow, olive flounder, rainbow trout, crucian carp and grass carp [25,28–31]. Like its mammalian orthologous, MAVS of some fish species has been verified to play important roles in the host cells against viral infection through inducing the expression of IFNs and ISGs [28,32].

Black carp (*Mylopharyngodon piceus*) is a species of cyprinid fish and the sole species of the genus *Mylopharyngodon*. Black carp is among the culturally important “four famous domestic fishes” for over a thousand years in China and is the most highly esteemed and expensive food fish among the four domestic fishes. This is the first report about the innate immune system of black carp although it is subjected to lots of pathogens in the natural environment, such as grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV). In this paper, the black carp MAVS (bcMAVS) was cloned and characterized. The mRNA expression of bcMAVS was determined in different tissues with or without GCRV or SVCV infection. The protein expression and intracellular location of bcMAVS was identified by immunoblotting (IB) and immunofluorescence assay (IF) separately. The induction of zebrafish IFN and EPC IFN by bcMAVS was determined by luciferase reporter assay. This study has built a solid foundation for the further study of how bcMAVS functions in the innate immune response against pathogen invasion in black carp.

2. Materials and methods

2.1. Cells and plasmids

HEK293T (293T) and NIH3T3 cells were kept in the lab. CIK (*C. idella* kidney) and EPC (epithelioma papulosum cyprini) cells were kind gifts from Dr. Pin Nie (Institute of Hydrobiology, CAS). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100u/ml penicillin and100ug/ml streptomycin.

The recombinant expression vector pcDNA5/FRT/TO-HA-bcMAVS was constructed by cloning the open reading frame (ORF) of black carp MAVS (bcMAVS) fused with an HA tag at its N-terminus into pcDNA5/FRT/TO (Invitrogen), and HA tag was fused at the C-terminus for pcDNA5/FRT/TO-bcMAVS-HA accordingly. RFP-HA-bcMAVS and EGFP-HA-bcMAVS were constructed by inserting HA-bcMAVS into Dsred-C1 and EGFP-C1 vector separately (Invitrogen). Mitochondrion indicator Mito-EGFP was a kind gift from Dr. Pinghui Feng from University of Southern California and used as the indicator of mitochondrion. pRL-TK, Luci-zIFN (for zebrafish interferon promoter activity analysis) and Luci-eIFN (for EPC interferon promoter activity analysis) were kind gifts from Dr. Yong'an Zhang (Institute of Hydrobiology, CAS).

2.2. Cloning of bcMAVS cDNA

Degenerate Primers (Table 1) were designed for amplifying bcMAVS cDNA basing on the MAVS sequences of *Cyprinus carpio* (HQ850440) , EPC (CAX48603) and Zebra fish (NM_001080584). Total RNA was isolated from the spleen of black carp and first-strand cDNA were synthesized by using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas). Rapid amplification of cDNA ends (RACE) was performed to obtain 5′UTR (untranslated region) and 3′UTR of bcMAVS cDNA by using 5′ Full RACE Kit and 3′ Full RACE kite separately (TaKaRa). The full-length cDNA of bcMAVS was cloned into pMD18-T vector and sequenced by Invitrogen.

2.3. Virus produce and titer

Spring viremia of carp virus (SVCV) and grass carp reovirus (GCRV) were kindly provided by Dr. Yong'an Zhang (Institute of Hydrobiology, CAS). SVCV and GCRV were propagated in EPC and CIK separately at 25 °C in the presence of 2% fetal bovine serum. Virus titers were determined by plaque forming assay on EPC and CIK cells separately as previously described [33].

2.4. Semi-quantitative RT-PCR

Black carp of six months (weight of 20 g) were injected intraperitoneally with GCRV (2.52×10^6 pfu/fish), SVCV (2.43×10^6 pfu/fish) or sterile PBS separately and cultured at 25 °C. The injected black carp were sacrificed at 33 h post injection and total RNA was isolated from tissues of gill, kidney, heart, intestine, liver, muscle, skin and spleen independently. The primers of BC-MAVS-RT-5' (1144) and BC-MAVS-RT-3' (1506) were used to detect bcMAVS mRNA expression in the above tissues (Table 1). The semi-quantitative RT-PCR program was: 94 °C for 5 min, then 30 cycles of 94 °C/30 s, 58 °C/30 s and 72 °C/30 s, finally extension at 72 °C for 5 min. The enhanced RT-PCR program (for detecting bcMAVS transcription in muscle) was: 94 °C for 5 min, then 40 cycles of 94 °C/30 s, 58 °C/30 s and 72 °C/30 s, finally extension at 72 °C for 5 min.

2.5. Immunoblotting

HEK293T cells were transfected with FRT/TO-HA-bcMAVS, FRT/TO-bcMAVS-HA, or empty vector separately. Transfected cells were harvested at 48 h post-transfection and lysed for immunoblot (IB) assay as previously described [34]. Briefly, whole cell lysates were isolated by 10% SDS-PAGE and transferred membrane was probed with anti-HA antibody (1:2000; Sigma). Proteins were visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma).

2.6. Glycosidase digestion and phosphatase digestion

HEK293T cells were transfected with FRT/TO-HA-bcMAVS and transfected cells were harvested at 48 h post-transfection. Lysed cells were divided into two aliquots, one applied for the treatment of CIP (Alkaline Phosphatase, Calf Intestinal/NEB) or PNGase F (NEB) according to the manufacturer's instructions and the other aliquot