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A structural perspective of the MAVS-regulatory mechanism on the mitochondrial outer membrane using bioluminescence resonance energy transfer

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ABSTRACT

In most eukaryotic cells, mitochondria have various essential roles for proper cell function, such as energy production, and in mammals mitochondria also act as a platform for antiviral innate immunity. Mitochondrial-mediated antiviral immunity depends on the activation of the cytoplasmic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) signaling pathway, and on the participation of mitochondrial antiviral signaling (MAVS), which is localized on the mitochondrial outer membrane. After RNA virus infection, RLRs translocate to the mitochondrial surface to interact with MAVS, and the adaptor protein undergoes a conformational change that is essential for downstream signaling, although its structural features are poorly understood. Here we examined the MAVS-regulatory mechanism on the mitochondrial outer membrane using bioluminescence resonance energy transfer (BRET) in live cells. Using a combination of BRET and functional analysis, we found that the activated MAVS conformation is a highly ordered oligomer, at least more than three molecules per complex unit on the membrane. Hepatitis C virus NS3/4A protease and mitofusin 2, which are known MAVS inhibitors, interfere with MAVS homotypic oligomerization in a distinct manner, each differentially altering the active conformation of MAVS. Our results reveal structural features underlying the precise regulation of MAVS signaling on the mitochondrial outer membrane, and may provide insight into other signaling systems involving organelles.

1. Introduction

RNA viruses gain entry into host cells either through endocytosis, where the plasma membrane engulfs the virion, resulting in the release of the virion constituents into the cell, or through membrane fusion with the plasma membrane, resulting in the immediate release of viral replication machinery and incorporation of the corresponding nucleic acids into the host cytoplasm. To counter the plasma membrane fusion of RNA viruses, vertebrates have evolved the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) pathway, which detects cytoplasmic viral-derived double-stranded (ds)RNA and induces a signaling cascade to initiate an antiviral innate immune response [1,2]. In humans, the RLRs pathway detects several viruses from five families (*Paramyxoviridae*, *Orthomyxoviridae*, *Rhabdoviridae*, *Flaviviridae*, and *Reoviridae*), which include hepatitis C, Dengue, Japanese encephalitis, rabies, and influenza A viruses [3,4].

The initial sensors of intracellular viral RNA are caspase activation and recruitment domain (CARD)-containing RNA-helicase proteins, RIG-I and melanoma differentiation-associated gene 5 (MDA5), which differ based on the size and type of the viral RNA [3–5]. Post-nucleotide recognition by the RLRs [6,7] leads to translocation of the RNA helicases to the scaffold adaptor protein, mitochondrial antiviral signaling (MAVS [8]; also known as IPS-1 [9], VISA [10], and Cardif [11]), a ubiquitously expressed mitochondrial outer membrane protein. Upon the upstream signaling event, MAVS is proposed to undergo a conformational switch that leads to the recruitment of various downstream effectors, such as tumor necrosis factor receptor-associated factor (TRAF) family members and tumor necrosis factor receptor-associated death domain (TRADD), to form a supramolecular signaling assembly. The mitochondrial hub of the protein complex, the so-called MAVS signalosome, is essential for upregulation of the transcriptional factors nuclear factor κB (NF-κB) and interferon regulatory factor-3 (IRF-3), leading to the rapid production of type I interferons (IFNs) and proinflammatory cytokines [12,13].

Because the prominent function of MAVS signaling is precisely regulated upon viral infection [13–15], it is crucial to understand

Abbreviations: BiFC, bimolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; CARD, caspase activation and recruitment domain; HCV, hepatitis C virus; HEK, human embryonic kidney; HR1, 4,3 hydrophobic heptad repeat region 1; IFN-β, interferon β; IRF-3, interferon regulatory factor 3; MAVS, mitochondrial antiviral signaling; Mfns, mitofusins; NF-κB, nuclear factor κB; NLRX1, NOD-like receptor family member X1; PRR, proline rich region; RIG-I, retinoic acid-inducible gene I; Rluc, *Renilla* luciferase; RLR, RIG-I-like receptor; Venus, a yellow fluorescent protein derivative

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how the conformational switch is regulated and the machinery involved in activating and inactivating MAVS signaling. In fact, these processes comprise transiently populated states during the signaling event, making it difficult to directly determine the structure of MAVS. Further, MAVS signaling occurs on the mitochondrial surface, so it is somewhat altered when reconstituted under isolated *in vitro* conditions. In the present study, we bypassed the practical issues mentioned above and investigated the MAVS-regulatory mechanism in live cells using a fluorescence-based assay combined with functional study. Our results indicated that MAVS undergoes a homotypic interaction, resulting in a highly oligomerized state toward signaling. The hepatitis C virus (HCV) protease NS3/4A, NOD-like receptor family member X1 (NLRX1), and mitofusin 2 (Mfn2), all of which are known MAVS inhibitors [12,13,15], differentially target the MAVS conformation to inhibit its actions. Based on these findings, we provide structural insight into mitochondrial-mediated antiviral signaling.

2. Materials and methods

2.1. Materials and cell cultures

The human embryonic kidney (HEK) 293 cell line was maintained in Dulbecco's modified Eagle medium (D-MEM; GIBCO BRL, Carlsbad, CA) supplemented with 1% L-glutamine, 1% penicillin–streptomycin, and 10% bovine calf serum (BCS) at 5% CO_2 and 37 °C. The rabbit polyclonal antibody against human MAVS was described previously [16]. Anti-Myc (9E10) and anti-HA (HA.11) monoclonal antibodies were purchased from Covance (Princeton, NJ), anti-phospho-IRF-3 (Ser396) rabbit monoclonal (4D4G), anti-Hsp60, and anti-COX IV polyclonal (D307) antibodies were obtained from Cell Signaling Technology (Danvers, MA), and anti-mtHsp70 monoclonal antibody was purchased from Affinity BioReagents (Golden, CO). Anti-β-actin, anti-Mfn2, and anti-Tom-20 monoclonal antibodies, as well as anti-Mfn1 and anti-cytochrome *c* polyclonal antibodies, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-GFP antibody was from MBL (Nagoya, Japan), and anti-OPA-1 monoclonal antibody was from BD Biosciences (San Jose, CA). Coelenterazine H was purchased from Promega (Madison, WI). The *NLRX1*-deficient mouse embryonic fibroblast (MEF) was kindly provided by Jenny Ting (University of North Carolina at Chapel Hill). All other reagents were of biochemical research grade.

2.2. Plasmid construction and mutagenesis

Polymerase chain reaction (PCR) was performed with PrimeSTAR DNA polymerase (Takara, Tokyo, Japan). The following oligonucleotide DNA primers were used to generate the complete open reading frames of the following genes:

human MAVS: 5′-aaaGCGGCCGCcatgccgtttgctgaagacaagacc (forward),
5′-tttGATATCtcagtgcagacgccgccggtacagcacc (reverse);
hMfn1: 5′-aaaGCTAGCaccATGGGGgcagaacctgtttctccac (forward),
5′-tttGGTACCggattcttcattgcttgaagg (reverse);
hMfn2: 5′-aaaGCTAGCaccATGGGGtccctgctcttctctcgatgc (forward),
5′-tttGGTACCtctgctgggctgcaggtactgg (reverse);
hNLRX1: 5′-aaGCGGCCGCcatgaggtggggccaccatttgcccagggcc (forward),
5′-ttGATATCtcagcttccagagcttcccagctgctccagg (reverse);
rat Tom-22: 5′-aaaGCGGCCGCcatggccgccgccgtcgctgc (forward),
5′-tttGATATCtcagatctttccaggaagtggagg (reverse);
murine Omp-25: 5′-aaGCGGCCGCcatgaacggacgggtgg (forward),
5′-tttGATATCtcaaagctgctttcggtatc (reverse);
Venus: 5′-aaGCTAGCaccatggtgagcaagggcg (forward; 1 A.A.),
5′-ttGCGGCCGCcttgtacagctcgtccatgccg (reverse; 239 A.A.),
5′-ttGCGGCCGCggcggtgatatagacgttg (reverse for Venus(N); 155 A.A.),
5′-aaGCTAGCaccatggacaagcagaagaacggc (forward for Venus(C); 156 A.A.);
Renilla luciferase (Rluc): 5′-aaGCTAGCaccatggcttccaaggtgtacgac cccgagc (forward),
5′-ttGCGGCCGCctgctcgttcttcagcacgcgctcc (reverse).

Fusion-tagged MAVS-expression plasmids were constructed by ligating the PCR amplimer into a pcDNA3.1 (−) vector (Invitrogen, Carlsbad, CA) that encoded either an N-terminal Venus or Rluc tag. Mutation (C508R) into Venus- or Rluc-MAVS plasmids was introduced by site-directed mutagenesis (Stratagene, La Jolla, CA). The Myc-tagged versions of MAVS, Mfn1, Mfn2, and their variants were described previously [16]. The NLRX1 variants were created by amplifying the regions comprising amino acids 1–975 (full length), 40–975 (mature), and 40–560 (NACHT) by using PCR, and ligating the PCR products into the pcDNA3.1 (−) vector that encoded either an N-terminal or C-terminal HA-tag. To generate retroviral expression constructs of NLRX1 variants, each HA-tagged NLRX1 cDNA was recloned into the retroviral vector pMXs-Puro (Cell Biolabs, San Diego, CA). The retroviral expression vectors were then transfected into the platinum packaging cell lines (Cell Biolabs), and the retroviral supernatant was harvested 48 h post-transfection and used to infect MEF cultures. All constructs used in the present study were confirmed by DNA sequencing (ABI 3100).

2.3. Dual luciferase reporter assay

Luciferase assays were performed as described previously [17] with a slight modification. In brief, HEK293 cells were plated in 24-well plates (2×10^5 cells/well). The following day, the cells were co-transfected with 50 ng of a luciferase reporter plasmid (p125luc, pELAM, or pISRE-Luc), 2.5 ng of Renilla luciferase internal control vector phRL-TK (Promega), and each of the indicated vectors using Lipofectamine 2000 reagent (Invitrogen). An empty vector [pcDNA3.1 (−)] was used to maintain equivalent amounts of DNA in each well. Cells were harvested 24 h post-transfection and analyzed by a dual-luciferase reporter assay on the GloMax 20/20n luminometer (Promega).

2.4. BRET saturation assay

All the BRET signals were measured using a Flexstation 3 Microplate Reader (Molecular Devices, Sunnyvale, CA) at 37 °C. For the assay, HEK293 cells were plated in 12-well plates (2.5×10^5 cells/well). The following day, the cells were co-transfected with a constant quantity (5 ng) of Rluc-tagged plasmid and increasing amounts of Venus-tagged constructs using Lipofectamine 2000. Empty vector [pcDNA3.1 (−)] was also used to maintain equivalent amounts of DNA in each well. Twenty hours post-transfection, the cells were washed once with 1× phosphate-buffered saline (PBS, pH 7.2), mechanically detached, and collected by centrifugation (800 *g* for 5 min). The cell pellets were then resuspended in 80 μL of Dulbecco's PBS (pH 7.2), and two 40-μL aliquots of cell suspensions were transferred to each well of white 96-well microplates (duplicate wells). After adding 10 μL of Rluc substrate (coelenterazine H, 25 μM) into each sample, followed by 30 s of gentle mixing, luminescence was measured simultaneously for the donor ($\lambda_{em}=475$ nm; short wavelength) and for the acceptor ($\lambda_{em}=530$ nm; long wavelength). Saturation data analysis was performed using the following equation, described previously [18]:

$$\text{BRET signal} = [\text{long wavelength}]/[\text{short wavelength}] - [\text{long wavelength}]_{\text{donor only}}/[\text{short wavelength}]_{\text{donor only}}.$$

2.5. Mitochondrial fractionation and proteolysis

HEK293 cells were washed once with cold 1× PBS (pH 7.2), scraped off the culture plate, and lysed in 800 μL homogenization