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The Mitochondrial Antiviral Signaling Protein, MAVS, Is Cleaved During Apoptosis

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Abstract

Apoptosis of virus-infected cells is one important host strategy used to limit viral infection. Recently a member of the innate immune signaling pathway, MAVS, was localized to mitochondria, an organelle important for apoptosis regulation. Here we investigate what role MAVS may play in apoptosis. Induction of cell death led to the rapid cleavage of MAVS, resulting in its release from the outer mitochondrial membrane. This cleavage is blocked in cells incubated with proteasome or caspase inhibitors. Transfection of synthetic viral dsRNA and dsDNA also led to cleavage of MAVS, indicating that this process may be important during infection. Preventing apoptosis by over-expression of anti-apoptotic Bcl-xL blocks MAVS cleavage, placing this process downstream of caspase activation in the apoptotic program.

Keywords

MAVS; IPS-1; Cardif; Apoptosis; Innate Immunity; Mitochondria; Cleavage; Proteasome; Caspase

Introduction

The innate immune system is the first line of defense against viral infection. Viral particles are sensed by host-cell receptors, which initiate signaling cascades leading to an antiviral response [1,2]. In the case of cytoplasmic infection, binding of viral dsRNA to the helicase domain of RIG-I or MDA-5 induces these proteins to interact with MAVS (also known as IPS-1/VISA/Cardif), a CARD domain protein located on the outer mitochondrial membrane (OMM) [3,4,5,6]. MAVS then activates kinases, such as TANK-binding kinase 1 (TBK-1) and IKK ϵ , which phosphorylate interferon regulatory factor 3 (IRF-3), finally resulting in the production of cytokines such as type I interferon [3,4,5,6].

A second strategy to protect against viral infection is the induction of apoptosis. Several viruses (including cytomegalovirus and human immunodeficiency virus-1,) produce proteins that interfere with the apoptotic machinery of the host, indicating that cells use this death pathway to limit viral replication and transmission [7]. Apoptosis is also linked to immunity through the 2–5A system, which consists of 2'–5' oligoadenylate synthetase (2'–5' OAS) and the endoribonuclease, RNase L [8]. dsRNA activates the 2–5A system, leading to the RNase L-

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based degradation of ribosomal RNAs and apoptosis in mammalian cells [9,10,11]. Importantly, cells lacking functional RNase L are protected from apoptosis [9], and are also deficient in the production of β -interferon (IFN- β) caused by viral infection [12], placing this system at the core of two antiviral strategies.

Mitochondria are key regulators of apoptosis, particularly in terms of the Bcl-2 family of proteins. This group contains both pro-apoptotic (e.g. Bax, Bak) and anti-apoptotic (e.g. Bcl-2, Bcl-xL) members, whose actions are focused on the OMM. Given the importance of mitochondria in regulating apoptosis, and the connection between apoptosis and innate immunity, the discovery that the RIG-I adaptor protein MAVS resides on the OMM is cause for interest, as it may provide a link between these two processes at the mitochondrial level [14]. In this study we show that induction of apoptosis leads to the rapid cleavage of MAVS and its disassociation from the OMM. This cleavage is abrogated in cells treated with proteasome- and caspase-inhibitors, and correlates with cleavage of the apoptosis marker poly (ADP) ribose polymerase (PARP). Expression of the anti-apoptotic protein Bcl-xL also blocks MAVS cleavage, indicating that cleavage occurs downstream of the caspase-activation stage of apoptosis.

Materials and Methods

Cells and Culture Conditions

HeLa cells were grown in DMEM (Invitrogen) supplemented with 10 % FBS (Gemini Bio-Products), 1 mM HEPES, 200 μ M l-glutamine, 1 % MEM non-essential amino acids and 100 μ M sodium pyruvate (all Invitrogen).

Reagents and Plasmids

MAVS was PCR amplified from GFP-MAVS [15] and inserted into of YFP-C1 (Clontech). CFP-Bcl-xL was created by inserting Bcl-xL into pECFP (Clontech) as per [16], while CFP-C1 and mito-dsRED were obtained from Clontech. Plasmids were transfected using Fugene 6 (Roche). Cells were treated with 10 μ M actinomycin D, 500 nM staurosporine, 100 μ M MG101, 20 μ M MG115, 200 μ M AEBSF (all Sigma), 2 μ M epoxomicin, 50 μ M zVAD-fmk or 10 μ M MG132 (all Calbiochem). Poly(I:C) and Poly(dAT:dAT) from Sigma were transfected with Lipofectamine 2000 (Invitrogen).

Western Blotting and Subcellular Fractionation

For western blotting of total proteins, cells were harvested from six-well plates using 0.5 % trypsin-EDTA (Invitrogen), washed in 1 X PBS and lysed in buffer containing 1 % Triton X-100, 25 mM HEPES/KOH and 300 mM NaCl. Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membrane. For subcellular fractionation, cells were scraped from plates in PBS-EDTA and lysed on ice in sucrose buffer by passage through 25-gauge syringe needles. Each sample was separated by SDS-PAGE, followed by transfer to nitrocellulose membrane as above.

Membranes were blocked in non-fat milk, and incubated with either anti-MAVS (Abcam), anti-PARP (Biomol), anti- β -Actin (Sigma) or anti-Tim23 (BD Biosciences.), followed by incubation with the appropriate HRP-conjugated secondary antibody and detection by the ECL chemiluminescence system (both Amersham).

Fluorescence Microscopy and Imaging

Cells were plated on Lab-Tek chamber slides (Nunc) and observed using a Zeiss LSM 510 confocal microscope (Carl Zeiss). Captured images were merged using ImageJ (NIH) and arranged using Adobe Photoshop.

Results

Induction of apoptosis leads to proteasome- and caspase-dependent cleavage of MAVS

HeLa cells were treated with the broad-based kinase inhibitor staurosporine (STS) or the transcription inhibitor actinomycin D (ActD) to induce cell death, then endogenous proteins were examined by Western blotting. In untreated or DMSO control cells, the MAVS protein appeared predominantly as a band of approximately 57 kD (Fig. 1A), in keeping with previous reports [3]. However, in cells incubated with STS or ActD, the majority of endogenous MAVS migrated as a single band of around 51–54 kD (Fig. 1A). To monitor the levels of apoptosis involved, protein samples were examined for cleavage of PARP. Like MAVS, cleavage of PARP was only evident in STS- and ActD-treated cells (Fig. 1A), indicating that the change in MAVS molecular weight was linked to apoptosis. Cleavage of MAVS and PARP displayed similar kinetics over the course of three hours of STS treatment (Fig. 1B).

To determine whether cellular proteases activated during apoptosis were involved in MAVS cleavage, ActD and STS were added to HeLa cells that had been incubated with protease inhibitors. Addition of the proteasome inhibitor MG132 potently inhibited the cleavage of MAVS relative to cells treated with apoptosis-inducers alone (Fig. 1C). The inhibitory effect of MG132 on MAVS cleavage was also apparent in the human colorectal cancer cell line HCT116 (data not shown). MG132 also limited the amount of PARP cleavage caused by ActD and STS, strengthening the correlation between apoptosis and MAVS cleavage (Fig. 1C).

We then examined whether the inhibitory effect of MG132 on MAVS cleavage was related to its effect on proteasomal function, or whether (as the block of PARP cleavage suggested) the chemical abrogated the effect of other cellular proteases. Addition of another three proteasome inhibitors (epoxomicin, MG101 and MG115) all limited the cleavage of MAVS by STS, whereas a general serine protease inhibitor, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), had no effect (Fig. 1D). To investigate whether blocking caspases could inhibit the cleavage of MAVS, the pan-caspase inhibitor zVAD-fmk was added to cells treated with ActD and STS. In both cases, the addition of zVAD-fmk blocked MAVS and PARP cleavage during apoptosis (Fig. 1E). Combining these data, it is concluded that MAVS is subject to cleavage during apoptosis, and that this requires either an intact proteasome or functioning caspases.

Synthetic dsRNA and dsDNA cause MAVS cleavage and apoptosis

Synthetic forms of short, double stranded nucleotides are frequently used to imitate the entry of viral dsRNA and dsDNA into cells [e.g. 2,20,21]. To determine the effect of viral nucleotide mimics on MAVS cleavage, Poly(I:C) – a synthetic form of dsRNA – was either incubated with, or transfected into, HeLa cells. After six hours, the addition of 10 $\mu\text{g ml}^{-1}$ Poly(I:C) to the culture medium had no obvious effect on either MAVS or PARP (Fig. 2A). However, when the same concentration of Poly(I:C) was transfected into HeLa cells, both MAVS and PARP underwent significant cleavage (Fig. 2A). As with ActD- and STS-induced apoptosis, this cleavage could be inhibited by the addition of MG132 (Fig. 2A). To ensure that this was not a dose-dependent effect, we checked whether cleavage occurred at other concentrations. Poly(I:C) was transfected at between 10 $\mu\text{g ml}^{-1}$ to 100 pg ml^{-1} , and after six hours, both MAVS and PARP cleavage was evident when the concentration was reduced one-hundred-fold to 100 ng ml^{-1} (Fig. 2B). This indicates that relatively low levels of Poly(I:C) can lead to apoptotic conditions, which has important consequences in terms of the immune response.

To observe whether contact with dsDNA also leads to MAVS cleavage, cells were incubated or transfected with the double-stranded DNA mimic, Poly(dAT:dAT). Like Poly(I:C), Poly(dAT:dAT) had no effect on MAVS or PARP cleavage when added to the culture medium.

However, transfection with $10 \mu\text{g ml}^{-1}$ Poly(dAT:dAT) led to cleavage of both proteins, albeit at a reduced level compared to the same concentration of Poly(I:C) (Fig. 2C). From these results, it would appear that both synthetic forms of double-stranded nucleotides can induce MAVS cleavage. However dsRNA has a greater effect when transfected at equivalent levels, possibly by its capacity to induce the RNase L system [9,12].

Cleavage of MAVS leads to its disassociation from the outer mitochondrial membrane

Several RNA viruses, such as Hepatitis A and C, disrupt the antiviral signaling pathway by cleaving MAVS from the mitochondrial membrane, which abolishes its ability to communicate with the downstream factors that lead to β -interferon production [6,22,23]. We speculated that the apoptotic cleavage of MAVS would also lead to its dislocation from mitochondria. To visualize MAVS *in vivo*, YFP-MAVS was co-transfected with mito-dsRED in HeLa cells and viewed by confocal microscopy. In both untreated and MG132-incubated cells, there was colocalization between YFP-MAVS and the mitochondrial marker, showing that MAVS is localized to mitochondria (Fig. 3A). In the majority of STS-treated cells, there is a marked difference in the localization of YFP-MAVS, as MAVS is no longer associated with mitochondria, and instead is found diffuse throughout the cytoplasm (Fig. 3A). The mitochondria themselves have changed morphology from long tubules, to short, punctate structures, as is usual in cells undergoing apoptosis [24]. Incubation of STS-treated cells with MG132 blocks the disassociation of MAVS from the mitochondria, and there remains a close colocalization between YFP-MAVS and mito-dsRED (Fig. 3A).

To ensure that the observed change in localization is related to MAVS and not the attached fluorescent protein, endogenous proteins were separated by subcellular fractionation. In untreated and MG132-incubated cells, MAVS was found exclusively in the membrane fraction, in agreement with the localization observed by confocal microscopy (Fig. 3B). After three hours, treatment of cells with STS led to the presence of the cleaved form of MAVS only, with no protein remaining attached to the mitochondrial membrane (Fig. 3B). The size of the cleavage product (around 51–54 kD), along with its presence in the cytosol, indicates that cleavage occurs at the extreme C-terminus of MAVS, near its transmembrane domain. Finally, in agreement with confocal data, addition of MG132 to STS-incubated cells reverses the change in MAVS localization, the majority being found in the membrane fraction as expected (Fig. 3B). In summary, the apoptotic cleavage of MAVS results in its detachment from the mitochondrial membrane.

Expression of apoptosis-inhibiting proteins reduces MAVS cleavage following apoptosis induction

The anti-apoptotic Bcl-2 family member, Bcl-xL, inhibits apoptosis by antagonizing the actions of Bax and Bak, preventing the permeabilization of the mitochondrial outer membrane and activation of caspases [13]. To examine whether over-expression of Bcl-xL could prevent the cleavage of MAVS following apoptosis induction, we expressed YFP-MAVS and CFP-Bcl-xL in HeLa cells treated with STS. In cells co-transfected with the control CFP-C1 plasmid, YFP-MAVS was found at the mitochondria in untreated cells, but in the cytoplasm in STS-treated cells three hours (Fig. 4A,B). In contrast, in the vast majority of cells expressing CFP-Bcl-xL, YFP-MAVS remained on the mitochondria in both STS treated and untreated cells, as shown by the co-localization of YFP-MAVS with the mitochondrial marker mito-dsRED (Fig. 4A,B). Similar results were obtained in HeLa cells over-expressing the anti-apoptotic proteins Bcl-2 and ν MIA (data not shown).

To examine whether these data reflected total protein content, we looked at the cleavage state of endogenous MAVS and PARP in HeLa cells over-expressing the control plasmid CFP-C1 and CFP-Bcl-xL. In control cells, the vast majority of endogenous MAVS and PARP were

cleaved in STS treated cells, and this was reversed to a large extent when MG132 was added to the culture medium (Fig. 4C). However, in cells expressing CFP-Bcl-xL, cleavage of MAVS and PARP was largely or completely absent in both STS- and STS- plus MG132-treated cells (Fig. 4C). These data indicate that the ability of Bcl-xL to prevent apoptosis also ablates the cleavage of MAVS in cells treated with apoptosis-inducing chemicals. As Bcl-xL acts at the start of the intrinsic apoptotic program, this would place the cleavage of MAVS downstream of the mitochondrial permeabilization and caspase activation stages of apoptosis.

Discussion

Cleavage of MAVS is a strategy used by several viruses to escape the host immune response and aid viral persistence. The NS3/4A protease of hepatitis C virus (HCV), and 3ABC protease of hepatitis A (HAV), cleave MAVS from the OMM at cysteine-508 and glutamine-428, respectively [6,22,23]. This blocks the interferon-signaling pathway initiated by binding of dsRNA to RIG-I or MDA-5, as MAVS function is dependent on its mitochondrial localization [3,23]. Mutagenesis of these sites prevents proteolytic cleavage, re-establishing normal MAVS function in HCV and HAV infected cells [6,22,23]. While apoptotic cleavage of MAVS appears to occur in the same C-terminal region as these sites, neither the Q428A HAV or C508A HCV mutations [6,23] were able to block STS-induced cleavage when introduced into YFP-MAVS (data not shown).

Cleavage of another protein in the interferon pathway, MDA-5, also occurs during viral infection [20]. Crucially, the same cleavage of MDA-5 is apparent in apoptotic cells, suggesting that MDA-5 is cleaved in response to the viral initiation of apoptosis. Like apoptotic MAVS cleavage (Fig. 1), degradation of MDA-5 is blocked by both proteasome and caspase inhibitors [20]. The similarity between the MDA-5 and MAVS cleavage scenarios suggests that inhibiting the function of these proteins, in this manner, may be a viral strategy to reduce the innate immune response. For example, cleavage of these proteins may abolish their signaling activity, lowering the production of cytokines, and aiding viral persistence.

The synthetic double-stranded nucleotide, Poly(I:C), is commonly used in studies to simulate the dsRNA produced during viral replication, including several reports on MAVS [3,4,5,21]. However, previous studies have shown that Poly(I:C) can activate the 2–5A system, stimulating the RNase L endoribonuclease and inducing apoptosis [9,10,11]. In this study, HeLa cells transfected with sub-micromolar levels of Poly(I:C) showed cleavage of both MAVS and PARP, indicating that cells had initiated an apoptotic program (Fig. 2). While this effect may be cell-type specific, it demonstrates a potential difficulty in using synthetic dsRNA in immunological studies, as Poly(I:C) has the ability to activate two intersecting pathways involved in the immune response.

The ability of both proteasome- and caspase-inhibitors to block MAVS cleavage (Fig. 1) indicates that both protease systems are required to effect degradation of the protein. However, while the chemical proteasome inhibitors used are generally thought to be specific, data presented here show that they are also able to ablate PARP cleavage, which is normally viewed as a substrate of caspase-3 [17,19]. This would indicate that proteasome inhibitors are either blocking apoptosis in general, or that they have the capacity to block the activation of caspase-3 specifically. Studies of the relationship between apoptosis and the proteasome have demonstrated a high level of interaction between the two. For example, regulatory proteins of the apoptotic machinery, such as p53, Bcl-2 family members, inhibitor of apoptosis proteins (IAPs) and Smac/DIABLO, are proteasomal substrates [25]. As such, it remains difficult to determine which pathway is initiated first during MAVS cleavage.

Despite the overlapping localization of Bcl-xL with MAVS (Fig. 4), there is no evidence thus far to show a direct interaction between them in the regulation of apoptosis. However, it is becoming clear that MAVS is likely to play a role in some aspects of apoptosis. An early report showed that siRNA depletion of MAVS in HEK293 cells led to cleavage of PARP [3], indicating that MAVS had a cytoprotective function. A subsequent study showed that MAVS siRNA in 293T cells blocked the induction of apoptosis due to reovirus infection, suggesting that MAVS is pro-apoptotic in these conditions [26]. These conflicting results indicate that, while MAVS is a candidate protein in the field of apoptosis regulation, we have much to learn regarding its function in this process.

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Figure 1. Induction of apoptosis leads to proteasome- and caspase-dependent MAVS cleavage
 HeLa cells were treated with 500 nM staurosporine (STS), or 10 μ M actinomycin D (ActD), for 3 h or 6 h respectively (A) or 500 nM STS only for 0 – 3 h (B). For inhibitor studies, HeLa cells were incubated as above with the addition of either 10 μ M MG132 (132; C), 200 μ M AEBSF (AEB), 2 μ M epoxomicin (Epox), 100 μ M MG101 (101), 20 μ M MG115 (115; all D) or 50 μ M zVAD-fmk (zVAD; E) were added to the culture medium either alone, or at the same time as either STS or ActD. DMSO (A) was added as a control to show effect of solvent only. After the noted time, cells extracts were analyzed by Western blot.

Figure 2. Synthetic dsRNA and dsDNA cause MAVS cleavage and apoptosis

HeLa cells were incubated (A, C), or transfected using Lipofectamine (Lipo; A–C), with Poly (I:C) or Poly(dAT:dAT). In (A), 10 μ g ml⁻¹ Poly(I:C) was added to the culture 15 medium or transfected into cells for h. In (B), Poly(I:C) was transfected into cells for 6 h at the indicated concentrations per ml. In (C), Poly(I:C) or Poly(dAT:dAT) were added to the culture medium or transfected into cells for 6 h at 10 μ g ml⁻¹. Transfections occurred in the presence or absence of 10 μ M MG132 as noted. After 6 h, cells extracts were analyzed by Western blot.

Figure 3. Subcellular localization of MAVS during apoptosis

The subcellular localization of YFP-MAVS (A) and endogenous MAVS (B) was analyzed by confocal microscopy and subcellular fractionation, respectively, in the presence or absence of 500 nM STS and/or 10 μ M MG132. In (A), YFP-MAVS and mito-dsRED were co-expressed in HeLa cells by transient transfection, followed by treatment with MG132 and/or STS for 3 h. Slides were analyzed by confocal microscopy and representative images for each treatment are shown. In (B), untransfected HeLa cells were treated as shown for 3 h, after which cells were harvested, lysed in sucrose buffer and split into membrane (M) and cytosolic (C) fractions by differential centrifugation. 25 μ g (each) of membrane and analyzed by Western blot.

Figure 4. Expression of anti-apoptotic proteins and MAVS cleavage

MAVS cleavage was examined in HeLa cells transiently expressing the anti-apoptotic Bcl-2 family protein Bcl-xL by confocal microscopy and Western analysis. In (A), YFP-MAVS, and mito-dsRED were co-transfected into HeLa cells, along with either CFP-C1 (control) or CFP-BclxL. Cells were incubated in culture medium with or without 500 nM STS for 3 h. In (B), cells transfected with YFP-MAVS, mito-dsRED and either CFP-C1 or CFP-Bcl-xL were incubated in culture medium with or without 500 nM STS for 3 h. The localization of YFP-MAVS was quantified in each case ($n = 50$), and independently repeated two further times ($n_{\text{total}} = 150$). In (C), wild-type HeLa cells expressing either empty CFP-C1 vector or CFP-Bcl-xL were treated with μM MG132 and/or 500 nM STS for 3 h. After 3 h, cells extracts were analyzed by Western blot.