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Research article

# Rabies virus matrix protein induces apoptosis by targeting mitochondria

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ABSTRACT

Apoptosis, as an innate antiviral defense, not only functions to limit viral replication by eliminating infected cells, but also contribute to viral dissemination, particularly at the late stages of infection. A highly neurotropic CVS strain of rabies virus induces apoptosis both *in vitro* and *in vivo*. However, the detailed mechanism of CVS-mediated neuronal apoptosis is not entirely clear. Here, we show that CVS induces apoptosis through mitochondrial pathway by dissipating mitochondrial membrane potential, release of cytochrome c and AIF. CVS blocks Bax activation at the early stages of infection; while M protein partially targets mitochondria and induces mitochondrial apoptosis at the late stages of infection. The α-helix structure spanning 67–79 amino acids of M protein is essential for mitochondrial targeting and induction of apoptosis. These results suggest that CVS functions on mitochondria to regulate apoptosis at different stages of infection, so as to for viral replication and dissemination.



## 1. Introduction

Apoptosis is an active process of cellular self-destruction which is considered as a host innate defense mechanism by eliminating virus-infected cells against rampant viral replication. However, apoptosis may also contribute to release of viral progeny and avoid the immune response. Therefore, many viruses have evolved numerous strategies to either induce or suppress apoptosis at different stages of infection for their own benefits [1].

Virus infection can trigger several cellular signaling pathways that converge at activation of caspases and ultimately result in apoptosis. Two major apoptotic pathways leading to caspase activation have been well described: the extrinsic pathway (death receptor pathway) which is initiated by cell surface receptor and mediated by activation of caspase-8, and the intrinsic pathway (mitochondrial pathway) which is regulated by mitochondria and mediated by activation of caspase-9 [2,3]. Both pathways eventually converge at the activation of caspase-3, which subsequently cleaves a variety of target substrates, such as poly (ADP-ribose) polymerase (PARP) and results in nuclear fragmentation [4].

Mitochondria are multifunctional organelles which not only play essential roles in host immune response but also serve as an important control point in the regulation of apoptosis. Following apoptotic signals, mitochondria undergo loss of mitochondrial membrane potential and subsequently release pro-apoptotic factors [5]. This process is highly regulated by Bcl-2 family proteins, which consist of both anti-apoptotic and pro-apoptotic family members, depending on the presence of at least one of four conserved Bcl-2 homology (BH) domains. Pro-apoptotic multi-domain proteins Bax and/or Bak are critical to the induction of mitochondrial apoptosis [6]. Upon activation, Bax undergoes conformational changes and results in Bax translocating to mitochondria, where Bax form oligomers resulting in loss of mitochondrial membrane potential [7]. While in healthy cells, Bax is primarily in the cytoplasm as inactive monomer and is held in check by the anti-apoptotic protein Bcl-2 [8]. The BH3-only proteins, such as Bad, which possess only the BH3 domain, function as upstream sensors of signaling pathways and convey to other Bcl-2

*Abbreviations:* RABV, rabies virus; CVS, challenge virus standard-11 strain of fixed rabies virus; VSV, vesicular stomatitis virus; AIF, apoptosis-inducing factor; cyto *c*, cytochrome *c*; PARP, poly (ADP-ribose) polymerase; LDH, lactate dehydrogenase; TOMM20, translocase of the outer mitochondrial membrane 20; CLSM, confocal laser scanning microscopy; SRM, super-resolution microscopy

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family proteins to initiate apoptosis. Upon activation, Bad subsequently activates Bax and Bak or inhibits the anti-apoptotic function of Bcl-2 and then leads to release of pro-apoptotic factors such as cytochrome *c* (cyto *c*) and apoptosis-inducing factor (AIF) [9]. Cyto *c* in the cytosol activates caspase-9 and then leads to irreversible cell death, which represents the mitochondrial caspase-dependent apoptotic pathway; AIF is proteolytically cleaved and truncated AIF is released from mitochondria and then translocated into the nucleus to induce high-molecular weight DNA fragmentation and chromatin condensation, causing the so-called caspase-independent cell death [10]. Since the mitochondria represent a central crossroad, where pro- and anti-apoptotic signals are integrated, numerous viruses have been reported to regulate cell apoptosis at the mitochondrial level [11].

Rabies virus (RABV), a member of the *lyssavirus* genus of *Rhabdoviridae* family, is the causative agent of rabies with severe neurological symptoms and almost 100% mortality [12]. The viral genome is a single non-segmented negative strand RNA and comprises five genes encoding viral proteins, namely nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the viral RNA polymerase (L). The N, P and L proteins, together with viral genomic RNA, form a helical nucleocapsid, which is surrounded by a membrane composed of host lipids and two viral proteins, the M and G proteins [13]. Previous reports have shown that RABV induced apoptosis *in vitro* and *in vivo* [14–17]. However, the molecular mechanisms of RABV-induced apoptosis are still not fully understood. In this study, we show that RABV M protein partially targets mitochondria and induces mitochondrial apoptosis through caspase-dependent and caspase-independent pathways at the late stages of infection.

## 2. Materials and methods

### 2.1. Cells and viruses

Mouse neuroblastoma N2a cells, baby hamster kidney (BHK-21) cells and Human embryonic kidney epithelial (HEK) 293T cells were cultured in Dulbecco modified Eagle medium (DMEM, Life technologies Gibco, Carlsbad, CA) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated fetal bovine serum (Gibco) at 37 °C with 5% $CO_2$. The challenge virus standard-11 strain of fixed rabies virus (CVS) was stored in our laboratory. Cells were infected with CVS at a multiplicity of infection (MOI) of 1 when monolayers had reached 80% confluence. After 1 h of viral absorption, cells were gently washed with PBS. Fresh medium was added and the cells were cultured for various periods at 37 °C. Virus preparations were titrated on N2a cells, and then stored at −80 °C.

### 2.2. Antibodies and reagents

Rabbit monoclonal antibodies (RabMAb) against AIF (ab32516), Prohibitin (ab75771), Cleaved PARP (ab32064), Bad (ab32445), mouse monoclonal antibody (MAb) against cyto *c* (ab13575), Bax6A7 (ab5714) and rabbit polyclonal antibody (PAb) against TOMM20 (ab78547) were all purchased from Abcam (Cambridge, MA, USA). RabMAb against Bak (D4E4) was purchased from Cell Signaling Technology. Rabbit PAb against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (R1210-1), Bax (M1312-3) and MAb against Bcl-2 were purchased from HuaAn Biotechnology Co. Ltd. (Hangzhou, China). Mouse MAb and rabbit PAb to N protein of RABV, as well as mouse PAb to M protein of RABV, were prepared in our laboratory. Caspase-9 inhibitor (z-LEHD-FMK, 218761) and caspase-8 inhibitorII (z-IETD-FMK, 218759) were purchased from Calbiochem (San Diego, CA). Caspase-3 inhibitor (Ac-DEVD-CHO, C1206) was purchased from Beyotime (Beijing, China). 4′, 6′-diamidine-2-phenylindole (DAPI) was purchased from Roche (Mannheim, Germany).

### 2.3. Lactate dehydrogenase (LDH) release assay

The amount of LDH released from the cells at different times after infection was measured by a Cytotox 96 nonradioactive cytotoxicity assay (Promega, Madison, Wis.) according to the manufacturer's instructions. All experiments were carried out in triplicate and conducted at least twice. Results were expressed as x-fold of control.

### 2.4. Cell viability assay

Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay (Beyotime, Beijing, China). Briefly, N2a cells were plated in 96-well plates at a density of $1 \times 10^5$ cells/cm$^2$ infected by CVS or not for different times. 10 μl CCK-8 was added to each well, incubated for 2 h at 37 °C, and then the absorbance was measured at 450 nm using automated ELISA reader (Bio-Tech Instrument, USA). All experiments were carried out in triplicate and conducted at least twice. Results were expressed as percentage of the infected group relative to the control group (set as 100%).

### 2.5. Measurement of ATP levels

Intracellular ATP levels were determined using an ATP Bioluminescence Assay kit (Beyotime, Beijing, China). Briefly, N2a cells cultured in 6-well plates were infected by CVS or not for different times. Cell culture media was aspirated and the cells were washed twice with PBS. Then lysis solution was added into each well to harvest the cellular ATP. The ATP levels were determined following the protocol of the assay kit. Results were expressed as percentage of control (set as 100%).

### 2.6. Caspase-3, -8, and -9 activities assay

Caspase-3, -8, and -9 activities were detected using the ApoAlert Caspase Fluorescent Assay Kits (Clontech, Palo Alto, CA) according to the manufacture's protocol. Briefly, cells were cultured in 6-well plates. At different time points, cells were collected by centrifugation at 400*g* for 5 min, and then resuspended in chilled cell lysis buffer on ice for 10 min. Cell lysates were centrifuged at maximum speed for 10 min at 4 °C, and then the supernatant was collected. After the reaction buffer and corresponding caspase substrate were added, the mixture was incubated at 37 °C for 60 min in a water bath. The substrates of caspase-3, -8, and -9 were DEVD-AFC, IETD-AFC, and LEHD-AMC, respectively. Fluorometric detection for caspase-3 and caspase-8 was measured at 400-nm excitation and 505-nm emission wavelengths; fluorometric detection for caspase-9 was measured at 380-nm excitation and 460-nm emission wavelengths. Activities are expressed as fold change over the activity of the uninfected control cells.

### 2.7. TUNEL assay

Apoptotic cells were examined and quantified using an *In situ* Cell Death Detection Kit (Roche, Philadelphia, PA, U.S.). In brief, cells were seeded in 48-well plates. After infection or transfection for different times, cells were fixed in 4% paraformaldehyde for 1 h at room temperature. After rinsing twice with PBS, cells were permeabilized using 0.1% Triton X-100 in 0.1% sodium citrate for 5 min on ice. Cells were then incubated with the TUNEL reaction mixture for 1 h at 37 °C in a humidified atmosphere in the dark. Cells were then rinsed three times with PBS and stained DAPI for