Calcium flux and calpain-mediated activation of the apoptosis-inducing factor contribute to enterovirus 71-induced apoptosis

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Enterovirus 71 (EV71) is a causative agent of an array of childhood diseases with severe neurological manifestations implicated. EV71 infection is known to induce caspase-dependent apoptosis in cell cultures and animal models. However, whether an alternative apoptotic pathway independent of caspase activation can be triggered by EV71 infection has not been explored. In this study, we showed that calcium (Ca\(^{2+}\))-activated calpains are capable of mediating caspase-independent pathway activation during EV71-induced apoptosis in HeLa cells. Results from subcellular fractionation analysis and confocal imaging indicated that during EV71 infection, apoptosis-inducing factor (AIF), a primary mediator of the caspase-independent pathway, became truncated and translocated from the mitochondrion to nucleus. This was accompanied by the release of cytochrome \(c\), and sharply decreased mitochondrial membrane potential. AIF knockdown data indicated significant protection against apoptotic cell death, with greater protection provided by the addition of a pan-caspase inhibitor. The Ca\(^{2+}\)-dependent, calpain isoforms 1 and 2, but not cathepsins, were proven crucial for the altered AIF behaviour as studied by the pharmacological inhibitor and the knockdown approaches. We then analysed Ca\(^{2+}\) dynamics in the infected cells and found elevated levels of mitochondrial Ca\(^{2+}\). Treatment with ruthenium red, a mitochondrial Ca\(^{2+}\) influx inhibitor, significantly blocked calpain activations and AIF cleavage. Our conclusion was that calpain activation via Ca\(^{2+}\) flux plays an essential role in eliciting an AIF-mediated, caspase-independent apoptotic pathway in EV71-infected cells. These findings should be useful for understanding the virus-induced cytopathology and the impact of Ca\(^{2+}\) homeostasis on EV71 infection.

INTRODUCTION

Apoptosis, a mode of programmed cell death, is morphologically characterized by the dramatic cell shrinkage, chromatin condensation and nuclear fragmentation (Galluzzi et al., 2007). Apoptosis can be triggered by the activation of caspases, a family of cysteine proteases, via the extrinsic and intrinsic pathways. The extrinsic pathway is mediated by death receptors that bind to ligands and trigger the intracellular assembly of a death-inducing signalling complex, a molecular platform for activating the caspase cascade emanating from caspase-8 (Scaffidi et al., 1998). On the other hand, the intrinsic pathway activation is manifested by mitochondrial outer membrane permeabilization (MOMP) that is frequently linked with reduced mitochondrial membrane potential (MMP), which facilitates the release of mitochondrial factors such as cytochrome \(c\) (Cyt\(c\)) into the cytosol and consequent activation of pro-caspase-9 (Green & Kroemer, 2004). The two pathways converge upon effector caspase activation (e.g. caspase-3, -6 and -7); this in turn triggers cleavages in a variety of target substrates, culminating in cell death (Danial & Korsmeyer, 2004; Galluzzi et al., 2007).

An alternative caspase-independent apoptotic pathway is mediated mainly by apoptosis-inducing factor (AIF) (Chu et al., 2005; Cregan et al., 2002), a flavoprotein anchored to the outer face of the mitochondrial inner membrane in...
healthy cells. Following some apoptotic stimuli, the proteolytic cleavage of a 62 kDa AIF to a 57 kDa form is mediated by calpains or cathepsins, as assessed in vitro and/or in some types of cell cultures. Truncated AIF is released to the cytosol and translocated to the nucleus, where it mediates chromatin condensation and large-scale DNA fragmentation (Bidere et al., 2003; Cao et al., 2007; Chwieralski et al., 2006; Otera et al., 2005; Susin et al., 1999; Yuste et al., 2005).

Calpains are a family of calcium ($\text{Ca}^{2+}$)-dependent cysteine proteases. Calpain 1 (µ-calpain) and calpain 2 (m-calpain) are ubiquitous isoforms that are respectively activated by micromolar and millimolar concentrations of $\text{Ca}^{2+}$ in vitro; both are localized at the cytosol and mitochondrion. Their activity has been implicated in apoptosis, as a long list of calpain substrates are associated with pro-apoptotic status (Goll et al., 2003; Smith & Schnellmann, 2012; Storr et al., 2011). Moreover, $\text{Ca}^{2+}$ is a key regulator of cell survival; sustained $\text{Ca}^{2+}$ elevation inside cells may activate the calpains following treatment with some anti-cancer chemicals, thus triggering apoptotic cell death (Hajnoczky et al., 2003; Norberg et al., 2008).

Enterovirus 71 (EV71) belongs to the genus Enterovirus of the family Picornaviridae. EV71 is an etiological agent associated with multiple childhood illnesses ranging from mild hand, foot and mouth disease to severe poliomyelitis-associated with multiple childhood illnesses ranging from mild hand, foot and mouth disease to severe poliomyelitis-disruption is known to play a key role in apoptosis during pathogenic process associated with several types of viral infections (Chami et al., 2006) including human immunodeficiency virus (HIV), hepatitis C virus, influenza virus (Benali-Furet et al., 2005; Haughey & Mattson, 2002; Ueda et al., 2010) and two phylogenetically related EVs, PV and CVB3 (Brisac et al., 2010; Campanella et al., 2004; Irurzun et al., 1995). However, whether the caspase-independent apoptotic pathway is activated and the potential association with $\text{Ca}^{2+}$ signal activation in EV71-infected cells has yet to be determined.

In the present study, we showed that AIF is proteolytically cleaved and translocated from the mitochondria to the nuclei, a scenario proved to be mediated by calpains activated by $\text{Ca}^{2+}$ influx into the mitochondria following EV71 infection. Complete identification of all apoptotic pathways triggered in the EV71-infected cells is central to understanding the resulting cytopathology. Such data can support efforts to develop new strategies for controlling EV71 infection and potentially other diseases associated with apoptotic cell death.

**RESULTS**

Infections by several serotypes of EVs, including EV71, are known to elicit caspase-dependent apoptosis and DNA fragmentation in various types of cell cultures (Belov et al., 2003; Carthy et al., 1998; Chang et al., 2004; Kuo et al., 2002; Li et al., 2002). To determine if a caspase-independent apoptotic pathway is activated during the course of EV71 infection, we investigated the behaviour of AIF, the main mediator of caspase-independent apoptosis (Chu et al., 2005; Cregan et al., 2002). By Western blot analyses, we quantified AIFs from mitochondrial fractions at the indicated time points with the intensity of each band representing a full-length AIF (62 kDa) divided by that of the corresponding mitochondrial protein (cytochrome oxidase subunit IV, COX IV; 17 kDa) as a loading control and compared to that of the mock-infected cells. Our results indicated a moderate loss of mitochondrial AIF to approximately 60% between 6 and 10 h post-infection (p.i.) and to approximately 40% at 12 h p.i. (Fig. 1a). With nucleus extracts using a nuclear protein (proliferating cell nuclear antigen, PCNA; 34 kDa) as a loading control, we observed that truncated AIF (57 kDa) appeared in the nuclei at 6 h p.i., with a significant elevation at 12 h p.i. (Fig. 1a). In addition, AIF translocation was detected by confocal analyses; the AIF image (red) was undetectable in the nuclei (blue) until 12 h p.i. by EV71 (green) (Fig. 1b). These results suggest that EV71 infection caused mitochondrial damage leading to AIF release.

Next, we evaluated MOMP by detecting spatial alterations in the subcellular localization of CytC during EV71 infection (Fig. 2a). Western blot analysis indicated initial CytC localization at the mitochondrial fraction of the mock-infected cells. CytC was detected in the cytosolic fraction with concomitant loss from the mitochondrial fraction at 6 h p.i.; a more pronounced loss was observed at 12 h p.i. (Fig. 2a). We then investigated whether loss of MMP occurred utilizing JC-1 dye staining; red-orange fluorescence is attributed to potential-dependent JC-1 aggregation in the mitochondria while green fluorescence becomes dominant following mitochondrial membrane depolarization. The ratio of red to green fluorescence signals served as an MMP parameter independent of the mitochondrial mass. A time-dependent MMP loss was detected by fluorescence microscopy (Fig. 2b) and quantified in terms of fluorescence intensity (Fig. 2c) as cells displaying green fluorescence gradually dominated over red-orange fluorescence toward the end of EV71 infection.

To elucidate the role that AIF plays in eliciting apoptosis and cell death, we applied the lentivirus-based, RNA interference (RNAi)-mediated knockdown (shAIF) to
achieve a significant decline in AIF expression (Table S1 and Fig. S1a, available in JGV Online). As a consequence, the level of apoptosis was evidently lowered (Fig. 3a), and the viability was elevated (Fig. 3b) in the EV71-infected cells using lentivirus-shLuc vector as a negative control for transduction. We next made use of a general caspase inhibitor z-VAD-fmk (z-VAD) to ascertain if AIF exerted its effect in a caspase-independent manner. Compared with results from treatment with shAIF or z-VAD alone, co-treatment with shAIF and z-VAD had significantly greater effects on reversing apoptosis and cell death (Fig. 3a, b), suggesting that AIF activity complemented caspase-mediated apoptosis in the EV71-infected cells. Results from a morphological analysis indicated that the levels of EV71-induced cytopathic effect characterized by rounded, refractile and shrunken cells were consistent with results from the use of various treatment combinations (Fig. 3c).

To identify AIF cleavage and translocation triggers in the context of EV71-infected HeLa cells, we employed specific inhibitors to calpains or cathepsins that have been described as processing AIF under various conditions (Bidère et al. 2003; Otera et al., 2005; Yuste et al., 2005). Treatment with calpeptin, an inhibitor of both calpain 1 and 2, but not CA-074 Me (cathepsin B inhibitor) or pepstatin A (cathepsin D inhibitor), hampered the truncation and translocation following infection (Fig. 4a). The result was substantiated by RNAi targeting calpain 1 or 2 (Table S1 and Fig. S1b, c), with knockdown of either calpain isoform resulting in a significant reduction in AIF truncation following EV71 infection (Fig. 4b). Further support was provided by confocal microscopy analyses indicating the abrogation of AIF translocation from the mitochondria to the nuclei by calpeptin or by knockdown of either calpain isoform (Fig. 4c). Similar AIF behaviour was found in the infected SK-N-SH neuroblastoma cells following the previous treatments (Fig. S2), thus negating the possibility that AIF activation is limited to one specific cell type.

We next proceeded with the mechanistic studies on calpain for consequent AIF release. Calpain 1 and 2 protein levels were investigated by a Western blot analysis, and found

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**Fig. 1.** Temporal and spatial alterations in AIF subcellular localization during EV71 infection. HeLa cells were mock-infected or infected with EV71 at an m.o.i. of 1. (a) At indicated time points, cells were subjected to subcellular fractionation for mitochondria and nucleus extracts, followed by a Western blot analysis with an antibody to AIF. All samples were run on the same gel. After protein transfer, the membrane was cut into strips and probed for the indicated protein. Arrows indicate full-length AIF (62 kDa) and truncated AIF (57 kDa). COX IV (17 kDa) and PCNA (34 kDa) served as markers for mitochondrial and nuclear proteins, respectively. (b) Confocal microscopy for the viral VP1 protein (green), AIF (red) and the nuclei (blue) in HeLa cells was used to observe cells either mock-infected (upper panels) or infected with the viral stock at an m.o.i. of 1 for 12 h (lower panels). Arrows in the images indicate nuclear translocation of AIF. Bar, 5 µm; M, mock-infected.
that in either case the level remained unaltered during the infection period (Fig. 5a). Since calpain activity can be upregulated regardless of protein level, we then employed a fluorogenic substrate-based assay to measure calpain activity in cell extracts. Progressive increases in calpain activity were observed throughout the EV71 infection period; this is substantiated by the findings that calpain activity was significantly reduced following the treatment with calpeptin or knockdown of either calpain isoform 12 h p.i. (Fig. 5b).

Fig. 2. Mitochondrial dysfunction in the EV71-infected cells. HeLa cells were mock-infected or infected with EV71 at an m.o.i. of 1 for 6 h and 12 h. (a) Cells were subjected to subcellular fractionation for mitochondrial and cytosolic extracts, followed by Western blot analyses with a polyclonal antibody to CytC (12 kDa). COX IV (17 kDa) and β-actin (43 kDa) served as markers for mitochondrial and cytosolic proteins, respectively. (b) Cells mock-infected (left panel), infected for 6 h (middle panel) and 12 h (right panel) were stained with JC-1 dye and observed with a fluorescence microscope. (c) JC-1 fluorescence was quantitatively measured with a fluorescence plate reader. The y-axis indicates the red/green fluorescence ratio of EV71-infected cells normalized to control cells, which is indicative of relative MMP. Data represent mean values of triplicate experiments, with each bar representing 1 SD (***P<0.001). Bar, 10 μm.

Fig. 3. AIF knockdown significantly reduced apoptotic cell death while elevating cell viability independent of caspase following EV71 infection. (a) HeLa cells were transduced with lentivirus-shAIF or -shLuc vector (a negative control for transduction) in the presence of DMSO (0.05%) or z-VAD (100 μM dissolved in 0.05% DMSO) for 36 h prior to infection with EV71 at an m.o.i. of 1 for 12 h. Treatment combinations were designated as groups I through V, as indicated. Cells were stained with Hoechst dye (0.5 μg ml⁻¹), and the apoptotic cells revealed by chromatin condensation or nucleus fragmentation were enumerated from more than 500 cells per condition. (b) Cell survival was measured using the MTS assay and expressed as the percentage of the mock-infected cells. Data were from three separate experiments, with means and SE shown (***P<0.001 and **P<0.01). (c) Cells were also observed with a phase-contrast microscope following treatments of groups I through to V. Bar, 5 μm.

fluorogenic substrate-based assay to measure calpain activity in cell extracts. Progressive increases in calpain activity were observed throughout the EV71 infection period; this is substantiated by the findings that calpain activity was significantly reduced following the treatment with calpeptin or knockdown of either calpain isoform 12 h p.i. (Fig. 5b).
Calpains are a family of Ca$^{2+}$-dependent proteases localized to mitochondria (Goll et al., 2003; Smith & Schnellmann, 2012). Since apoptosis can be triggered by mitochondrial Ca$^{2+}$ overload (Green & Kroemer, 2004; Hajnoczy et al., 2003; Tsujimoto & Shimizu, 2007), we investigated whether EV71 infection resulted in Ca$^{2+}$ influx into the mitochondria. EV71-infected cells were stained with Rhod2-AM fluorescent dye to indicate Ca$^{2+}$ concentrations in the mitochondria at 4, 8 and 12 h p.i. The infected cells displayed progressive elevation in the Ca$^{2+}$ concentration.

**Fig. 4.** AIF cleavage and translocation in EV71-infected HeLa cells were inhibited by calpains, but not by cathepsins. (a) HeLa cells were treated with CA-074 Me (an inhibitor to cathepsin B; 10 μM), pepstatin A (an inhibitor to cathepsin D; 10 μM), calpeptin (an inhibitor to calpains; 80 μM) or DMSO (as a negative control; 0.05 %) 30 min prior to infection by EV71 at an m.o.i. of 1 for 12 h. Mitochondrial (COX IV; 17 kDa) and nuclear fractions (PCNA; 34 kDa) were prepared and analysed by Western blotting on AIF. (b) HeLa cells were transduced with lentivirus-based shCAPN1 or shCAPN2 (targeting calpain 1 or 2, respectively) for 36 h prior to infection with EV71 at an m.o.i. of 1 for 12 h. Cells transduced with the lentivirus-shLuc vector were used as a negative control. Cells were also treated with calpeptin (80 μM) 30 min prior to infection with EV71 at an m.o.i. of 1 for 12 h. Additionally, cells were transduced with lentivirus-shCAPN1, -shCAPN2 or -shLuc (as a negative control) vector 36 h prior to infection with EV71 at 1 m.o.i. for 12 h. Cell lysates were prepared and subjected to a fluorogenic substrate-based calpain activity assay. Calpain activity was expressed as relative fluorescence unit (RFU) per 50 μg protein lysate, relative to that of the mocked-infected cells (set at 1). Three independent experiments were conducted with the means and SD shown (***P<0.001). M, Mock-infected.

**Fig. 5.** EV71 infection activated the endogenous calpain 1 and 2 activities without elevating their protein levels. (a) HeLa cells were inoculated with EV71 stock at an m.o.i. of 1 for the indicated time period. Western blot analysis was conducted for calpain 1 (CAPN1; 76 kDa) and 2 (CAPN2; 76 kDa) using α-tubulin (55 kDa) as a loading control. (b) Cells were inoculated with an EV71 stock at an m.o.i. of 1 in the presence of DMSO (0.05 %) for 4, 8 and 12 h or left mock-infected. Cells were also treated with calpeptin (80 μM) 30 min prior to infection with EV71 at an m.o.i. of 1 for 12 h. Cell lysates were prepared and subjected to a fluorogenic substrate-based calpain activity assay. Calpain activity was expressed as relative fluorescence unit (RFU) per 50 μg protein lysate, relative to that of the mocked-infected cells (set at 1). Three independent experiments were conducted with the means and SD shown (***P<0.001). M, Mock-infected.
mitochondrial Ca\(^{2+}\) during the infection (Fig. 6a). To correlate increased mitochondrial Ca\(^{2+}\) with caspase-independent pathway activation, we used ruthenium red (RR), an inhibitor of the mitochondrial Ca\(^{2+}\) uptake uniporter (Zazueta et al., 1999). Mitochondrial Ca\(^{2+}\) concentration was significantly reversed 12 h following RR treatment in the context of EV71 infection (Fig. 6a); this was accompanied by a significant decline in calpain activity (Fig. 6b) and AIF cleavage inhibition (Fig. 6c). Combined, these data suggest that Ca\(^{2+}\) influx into mitochondria mediates the activation of the caspase-independent pathway by promoting calpain activity.

**DISCUSSION**

Apoptosis is an active, non-inflammatory and energy-dependent cell death process in response to a wide variety of stimuli, including EV71 infection. This process may represent an important step in progeny virion release and dissemination while limiting inflammatory and immune responses. Therefore, a better understanding of the molecular mechanisms underlying apoptosis caused by EV71 infection should be of benefit to elucidating the viral pathogenesis and the development of therapeutic approaches.

Indeed, caspase activations via the intrinsic and extrinsic pathways have been reported in cells infected by EVs, including EV71 (Belov et al., 2003; Carty et al., 1998; Chang et al., 2004; Kuo et al., 2002; Li et al., 2002). To our knowledge, this is the first report of AIF-mediated, caspase-independent apoptosis via Ca\(^{2+}\)-dependent calpain activation in the EV71-infected cells elicited at a level comparable to that resulting from a caspase-dependent mode.

The results reported here demonstrate that the apoptotic cell death induced by EV71 was not completely inhibited by z- VAD, a pan-caspase inhibitor; with AIF mediating the induced apoptosis, as supported by the knockdown study (Fig. 3). The effect of AIF knockdown on the levels of apoptosis and cell viability was comparable, if not greater, to that of a general caspase inhibitor (Fig. 3), thereby stressing the importance of AIF-mediated apoptosis. Our observation of AIF release from mitochondria (Fig. 1) is consistent with the data indicating that EV71 infection caused an efflux of CytC from mitochondria (Fig. 2a; Belov et al., 2003; Carty et al., 1998; Chang et al., 2004); both occurred subsequently to or in line with MOMP (Fig. 2b; Green & Kroemer, 2004; Tsujimoto & Shimizu, 2007). However, while CytC remained intact upon the release, AIF was released in a truncated form. Moreover, evidence is accumulating regarding the important role of AIF in neuronal death in an animal model (Wang et al., 2003) and in neuronal cell cultures where AIF knockdown or microinjection of a neutralizing antibody significantly reduces apoptosis in response to diverse stimuli (Chu et al., 2005; Cregan et al., 2002; Culmsee et al., 2005; Susin et al., 1999; Wang et al., 2004). Taken together, the mitochondrial release of AIF in the EV71-infected cells reported here may be mechanistically linked to the clinical neuropathogenicity caused by EV71 infection (Chang et al., 1999; McMinn et al., 2001).

Calpains are a group of Ca\(^{2+}\)-activated cysteine proteases with two ubiquitous calpain isoforms, 1 and 2, located in the cytosol and mitochondria (Smith & Schnellmann, 2012). Previous studies indicated that calpain 1 but not calpain 2 is involved in AIF cleavage in isolated rat liver cells (Ozaki et al., 2009), and that calpain 1 is not capable of cleaving AIF in mitochondrial fractions from SH-SY5Y neuroblastoma cells (Joshi et al., 2009). In this study, we found that both calpain 1 and 2 mediated AIF activation in two distinct cell lines (Fig. 4 and Fig. S2) contrary to the assumption of cell type-specific AIF activation. We also ruled out cathepsin involvement in AIF cleavage and translocation (Fig. 4), a scenario that has been reported to occur in adult mouse brain (Yuste et al., 2005). While it is unlikely that the mechanism underlying mitochondrial cleavage and AIF release in the EV71-infected cells is cell type-specific, it remains to be determined if it is stimulus-specific. Increase in calpain activity has been documented in various types of cells infected by different viruses, but with diverse consequences. Both calpain 1 and 2 are essential for the genome replication of echovirus 1, a phylogenetically close enterovirus, in the human SAOS-2.
osteoarcoma cell line (Upla et al., 2008). Infection of CVBs may elicit calpain 2- but not calpain 1-dependent entry into polarized endothelial monolayers and induce necrosis rather than apoptosis in the polarized intestinal epithelial cells (Boyzm et al., 2010, 2011). Additionally, CVB4 infection has been reported as activating calpain activity for autophagy induction in rat primary neurons (Yoon et al., 2008). On the other hand, calpain is activated by hepatitis C virus proteins, resulting in the inhibition of the extrinsic apoptotic pathway in hepatic cell lines (Simonin et al., 2009). The calpain system is, therefore, implicated in various functions in distinct cell types following infection by either phylogenetically remote or close viruses. Altogether, viruses have evolved to activate the host calpain system for their own benefit, in this case to trigger apoptosis presumably for viral pathogenesis.

Several viruses encode functions that modify Ca2+ signals, thus allowing them to hijack cellular functions to serve one or more steps of infection (Chami et al., 2006). In the case of EVs, it has been reported that PV-infected cells have increased cytosolic Ca2+ concentration (Irurzun et al., 1995), and that Ca2+ flux between the endoplasmic reticulum (ER) and the mitochondrion contributes to apoptosis provoked by PV infection (Brisac et al., 2010). In the present study, we further showed that mitochondrial Ca2+ uptake via the Ca2+ uniporter plays an important role in calpain activation and AIF processing (Fig. 5a, b). Ca2+ homeostasis has been reported as being regulated by and correlated with anti-apoptotic status by the EV 2B protein that perturbs the ER-mitochondrial Ca2+ signal (Campanella et al., 2004). However, the identities of one or more viral proteins that induce apoptosis via a Ca2+ signal and calpain activation are not yet known, although the PV 2BC increases cytosolic Ca2+ (Aldabe et al., 1997). Finally, since calpains have already been investigated as potential therapeutic targets in a variety of human diseases (Saez et al., 2006; Storr et al., 2011), the results addressed herein may provide new perspectives for therapeutic strategies for controlling EV71 infections and their consequences.

**METHODS**

**Cell cultures, virus and reagents.** HeLa (ATCC CCL-2) and SK-N-SH (ATCC HTB-11) cells were cultured in minimum essential medium (MEM) (Gibco–BRL) supplemented with 10 % FBS. EV71 (BrCr strain, ATCC VR784) was propagated in HeLa cells cultured with MEM supplemented with 2 % FBS (MEM-2) and titrated on a Vero cell monolayer by a plaque assay. 293T cells were a kind gift from Dr Ann-Ping Tsou. Chemicals used were 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Sigma–Aldrich), calpeptin, (Santa Cruz), CA-074 Me (Sigma–Aldrich), pepstatin A (Sigma–Aldrich), z-VAD-fmk (z-VAD; Calbiochem), Rhod2-AM (Enzo Life Sciences) and ruthenium red (RR; Sigma–Aldrich). All chemicals except RR were dissolved in DMSO (final concentration 0.05%).

**Subcellular fractionation.** Mitochondrion-, cytosol- and nuclear-enriched fractions were prepared by the Subcellular Proteome Extraction Kit (ProteoExtract, Calbiochem) following the manufacturer’s instructions.

**Western blot analysis.** Cell extracts were prepared, separated by SDS-gel electrophoresis, and transferred onto nitrocellulose membranes as previously described (Lu et al., 2004). Blots were incubated with rabbit anti-AIF polyclonal antibody (PAb) (1 : 200; Santa Cruz), rabbit anti-Cy3C PAb (1 : 500; Santa Cruz), rabbit anti-calpain 1 PAb (1 : 1000; Abcam), rabbit anti-calpain 2 PAb (1 : 1000; Abcam), mouse anti-COX IV MAb (1 : 100; Santa Cruz), mouse anti-PCNA MAb (1 : 100; Santa Cruz), mouse anti-actin MAb (1 : 1000; Chemicon) or mouse anti-α tubulin MAb (1 : 10000; Abnova) as a primary antibody, followed by incubation with HRP-conjugated goat anti-rabbit (1 : 2000; Abnova) or goat anti-mouse IgG (1 : 3000; Abnova) as a secondary antibody. Proteins were detected using chemiluminescent substrate (ECL).

**Confocal fluorescence microscopy.** EV71-infected HeLa cells grown in 24-well dishes were fixed with 4 % paraformaldehyde and penetrated by 0.2 % Triton X-100. Rabbit anti-AIF PAb (1 : 50; Santa Cruz) and mouse anti-EV71 VP1 MAb (1 : 1000; Chemicon) were used as primary antibodies. Rhodamine conjugated goat anti-rabbit IgG (1 : 100; Jackson) and FITC conjugated goat anti-mouse IgG (1 : 100; Jackson) were used as corresponding secondary antibodies. Cells were counterstained with Hoechst 33258 (0.5 μg ml−1; Sigma) for 10 min at room temperature to indicate nucleus location. FITC (green) and Rhodamine (red) emissions were confocally monitored using a laser scanning microscope combination system (Leica SP2).

**Determination of MMP.** EV71-infected HeLa cells were cultured in MEM-2 supplemented with 5 μM JC-1 at 37 °C for 30 min. Fluorescence was observed with a fluorescence microscope (Nikon TE200), and intensities quantitatively determined using a fluorometer plate reader (Infinite M200, TECAN) with a 488 nm excitation wavelength and 530 (green) and 590 (red) nm emission wavelengths.

**RNAi vector generation.** The lentivirus-based, short hairpin RNA (shRNA) constructs pLKO-shAIF, pLKO-shCAPN1 and pLKOsh-CAPN2, targeting human AIF, calpain 1 and calpain 2, respectively, and the negative control phLKO-shLuc targeting firefly luciferase (Table S1) were obtained from the National RNAi Core Facility, Academia Sinica, Taiwan. For lentivirus preparation, 293T cells were cotransfected with a pLKO-shRNA construct and two helper plasmids, VSV-G pMD.G and pCMVΔR8.91, with Lipofectamine 2000 reagent (Invitrogen). Cell supernatants containing recombinant lentivirus were collected at 48–72 h post-transfection, and virus titres were determined in HeLa cells by a cell viability assay (relative infectious unit method) according to the instructions of the National RNAi Core (http://rmiigen.sinica.edu.tw/file/protocol/4_1_EstimationLentinivirusTiterRIUV1.pdf). HeLa target cells were transduced at a m.o.i. at 3–5 in the presence of 8 μg ml−1 polybrene (Sigma).

**Apoptotic cell detection and cell viability assay.** Cells were stained with Hoechst 33258 (0.5 μg ml−1) for 10 min at room temperature. Numbers of apoptotic cells characterized by chromatin condensation or nuclear fragmentation were counted from populations of more than 500 cells using a fluorescence microscope (Nikon TE200) equipped with a UV filter. Cell viability was determined based on the described method (Lu et al., 2004) using the CellTiter 96 AQuinoCell Proliferation Assay (Promega) according to the manufacturer’s instructions.

**Calpain activity assay.** Calpain activity was measured using a calpain activity assay kit (Biovision) according to the manufacturer’s instructions. Brieﬂy, cells were lysed and clarified lysates incubated with a fluorogenic substrate (Ac-LLL-AFC) and reaction buffer for 1 h at 37 °C in darkness. Following ﬂuorogenic substrate cleavage, ﬂuorogenic portions emitted yellow-green fluorescence at a wave-
length of 505 nm following excitation at 400 nm, as measured using a fluorimeter apparatus (Infinite M200, TECAN).

**Mitochondrial Ca²⁺ accumulation detection.** Mitochondrial Ca²⁺ was measured using the cationic Rhod2-AM, known to trigger potential-driven uptake into the mitochondria. Once inside a matrix, mitochondrial esterases cleave acetoxyethyl (AM) ester to liberate the Rhod 2 indicator, which displays high intensity fluorescence after binding with Ca²⁺ (Brisac et al., 2010). Briefly, EV71 infected or mock-infected cells were incubated in MEM-2 supplemented with 5 μM Rhod2-AM for 30 min, washed with preheated (37°C) HT buffer (Campanella et al., 2004), supplied with preheated HT buffer (with 1 mM CaCl₂ supplemented) and incubated at 37°C for 30 min. Cells were observed with a Nikon TE2000 fluorescence microscope (excitation wavelength, 455 ± 25 nm; emission wavelength, 600 ± 25 nm).

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