Japanese encephalitis virus infection activates caspase-8 and -9 in a FADD-independent and mitochondrion-dependent manner

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Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, replicates primarily at the endoplasmic reticulum and thereby triggers apoptosis of infected cells. This study investigated the hierarchical activation of the caspase network induced by JEV infection. It was found that JEV activated the initiators caspase-8 and -9, as well as effector caspase-3, in infected baby hamster kidney and mouse neuroblastoma (N18) cells. In neuronal N18 cells, JEV infection triggered cytochrome c release from mitochondria, which in turn activated caspase-9 and -3. Treatment of JEV-infected N18 cells with cyclosporin A or ruthenium red, which attenuate mitochondrial injuries, blocked activation of caspase-9 or -3, typifying that, in neuronal cells, this apoptosis involves the mitochondrial pathway. Alternatively, in caspase-3-deficient MCF-7 cells, JEV persisted and readily triggered a typical apoptotic response, including cytochrome c release and full activation of caspase-9 and -8 along with caspase-6, indicating that JEV did not require caspase-3 to manifest caspase-8 activation and apoptosis. Interestingly, a Fas-associated death-domain-containing protein (FADD) dominant-negative mutant, which interfered with transmission of the extracellular death signals into cells through the Fas/tumour necrosis factor (TNF) receptor, failed to block JEV-induced apoptosis and caspase-8 activation, implying that receptor oligomerization of the Fas/TNF pathway might not participate in JEV-induced apoptosis. Taken together, these results illustrate that JEV infection triggers caspase cascades involving the initiators caspase-8 and -9, probably through FADD-independent but mitochondrion-dependent pathways.

INTRODUCTION

Caspases are the central executioners for most apoptotic responses when activated in proteolytic cascades (Cohen, 1997; Villa et al., 1997). Apoptosis is implemented by effector caspases, including caspase-3, -6 and -7, which cleave a variety of essential cellular targets, and their activations are controlled hierarchically by initiator caspases such as caspase-8 and -9. Caspase-8 activation is triggered by an extracellular death-receptor signalling pathway involving the Fas/tumour necrosis factor receptor (TNFR), whilst caspase-9 is activated by intracellular apoptotic stimuli through a mitochondrial pathway. The mitochondrial apoptosis pathway is initiated by holocytochrome c (Cyto-c) released from mitochondria, which causes apoptosome formation and in turn triggers caspase-9 autoactivation (Adams & Cory, 2002; Green & Kroemer, 2004). The death-receptor pathway, via CD95/Fas/Apo1 or TNFR, is activated through oligomerization of membranous receptors that recruit procaspase-8 and Fas-associated death-domain-containing protein (FADD) to form a death-inducing signalling complex (DISC). This induced proximity to DISC formation subsequently stimulates caspase-8 autoactivation (Chen & Wang, 2002; Muzio et al., 1998). These two pathways function separately in response to diverse death signals, but may converge to the same downstream effector caspases, caspase-3, -6 and -7 (Cohen, 1997). However, the death-receptor pathway has been reported to potentiate the mitochondrial pathway by triggering Cyto-c release through caspase-8-cleaved Bid protein, a pro-apoptotic member of the Bcl-2 family that targets mitochondria (Li et al., 1998). Caspase-3 and -7 may also participate directly in causing Cyto-c release from...
mitochondria in a feedback-regulation manner (Lakhani et al., 2006). Crosstalk of this kind between the two pathways further ensures that the given cell will execute the apoptotic process begun by the death stimulus.

Bcl-2 family members are crucial cellular mediators that modulate the outer-membrane permeability of mitochondria in most apoptotic pathways (Cory & Adams, 2002). As pro-apoptotic signals to mitochondria, the bcl-2 homologue (BH)-3-containing Bid and Bcl-X₁ lead to release of apoptogenic factors from the intermembrane space of mitochondria, including Cyto-c, apoptotic-inducing factor, endonuclease G, Smac/Diablo and Omi/HtrA2 (Green & Kroemer, 2004; Saelens et al., 2004). In contrast, BH1- and BH2-containing anti-apoptotic proteins, such as Bcl-2 and Bcl-X₁, inhibit mitochondrial permeabilization by forming heterodimers with Bak or Bax and thereby stabilize the permeability transition (PT) pore complex on mitochondria (Green & Kroemer, 2004; Saelens et al., 2004). Vertebrate bcl-2 was the first cellular gene to be recognized as blocking apoptosis induced by infection of certain RNA viruses (Duncan et al., 1999; Grandgirard et al., 1998; Liao et al., 1998; Rodgers et al., 1997; Ubol et al., 1994). Moreover, studies involving Sindbis virus (Levine et al., 1993), Semliki Forest virus (Scallan et al., 1997) and influenza virus (Hinshaw et al., 1994) have demonstrated that constitutive bcl-2 expression can prevent infected cells from undergoing apoptosis and help them to become persistently infected. These observations suggest that Bcl-2 plays a role in forcing an acute cytolytic RNA virus to infect its host cells chronically.

Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, is transmitted to humans through chronically infected mosquitoes. JEV is a positive-sense, single-stranded RNA virus that replicates primarily in the cytoplasm of infected cells. The cytopathic effect (CPE) of this encephalitis may result from the cell death of JEV-infected astrocytes and neuronal cells, or from infiltrated inflammatory cells caused by the cytokines released from infected astrocytes (Chen et al., 2000, 2004; Liao et al., 2002). JEV infection triggers apoptosis in numerous culture cell lines, such as baby hamster kidney (BHK-21) cells, mouse neuronal N18 cells and human neural precursor NT-2 cells (Liao et al., 1997). Overexpression of bcl-2 can effectively delay JEV-induced cell death and subsequently convert some target cells into persistently infected cells (Liao et al., 1997, 1998): as the functional Bcl2 is primarily located on the mitochondrial membrane, these observations suggest that JEV-induced apoptosis may involve the mitochondrial apoptosis pathway. Enforced bcl-2 expression does not, however, affect JEV replication and spread during the early lytic infection. Caspase-3-cleaved Bcl-2 products have been found in apoptotic cells, suggesting that caspase-3 activation may participate in JEV-induced apoptosis (Liao et al., 1998). In this study, we further characterized caspase activation cascades during JEV-induced apoptosis, focusing on activation of the initiators caspase-8 and -9, and the downstream executioners caspase-3 and -6. We found that JEV infection appeared to activate caspase-8 by a FADD-independent manner and to turn on caspase-9 by the mitochondrion-dependent pathway. Although the addition of caspase inhibitors diminished cell injury, they failed to block JEV replication, illustrating that completion of the JEV life cycle does not require caspase activation.

**METHODS**

**Viruses and cell lines.** The neurovirulent RP-9 strain of JEV (Chen et al., 1996) was used in this study. Mouse neuroblastoma N18 cells were grown in RPMI 1640 containing 10% fetal bovine serum (FBS; Gibco). The human breast epithelial adenocarcinoma cell line MCF-7 (ATCC HTB-22) was cultured in high-glucose Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% FBS. Virus propagation was carried out in BHK-21 cells and virus titres and the virus growth curve were determined by plaque assay on BHK-21 cells (Chen et al., 1996). Virus titres were determined as p.f.u. ml⁻¹. Virus at an m.o.i. of 5 was used to synchronize infection and virus titres correlated linearly with the number of target cells infected by JEV (Liao et al., 1997).

**Antibodies, reagents and plasmids.** Anti-Cyto-c antibody was purchased from Pharmingen, anti-CPP32 and anti-PARP antibodies were purchased from Santa Cruz and anti-actin antibody was purchased from Chemicon. All cysteine protease inhibitors were purchased from Santa Cruz and anti-actin antibody was purchased from Pharmingen, anti-CPP32 and anti-PARP antibodies were purchased from Chemicon. All cysteine protease inhibitors were purchased from Clontech and Bachem. Western blotting was performed as described previously (Su et al., 2002). Cyclosporin A (CsA) and ruthenium red (RR) were obtained from Sigma. Plasmids containing bcl-2 and its mutant D34A were a kind gift from D. E. Griffen and J. M. Hardwick (Cheng et al., 1997; Clem et al., 1998). The plasmids carrying c-FLIP (Yeh et al., 1998) and FADD dominant-negative mutant (FADD-DN) (Chen & Lai, 2001; Walsh et al., 1998) were obtained from M.-Z. Lai (Academic Sinica, Taipei, Taiwan) and V. Dixit (Genentech, San Francisco, USA), respectively. bcl-2–IRES–DsRed and D34A–IRES–DsRed were constructed by using the backbone of the pCDNA4-HisMax vector (Invitrogen).

**Preparation of subcellular fractions.** Preparation of cytosolic fractions from N18 and BHK-21 cells was as described previously (Pastorino et al., 1998) with modifications. Briefly, cells were disrupted and the nuclei pellets were centrifuged to separate the mitochondrial fraction. Supernatants were collected by centrifugation at 100 000 g for 60 min at 4 °C to obtain the cytosolic S-100 fraction.

**Apoptotic cell death analysis.** Cells grown to approximately 80% confluence were infected by JEV at an m.o.i. of 5, and flow cytometry using propidium iodide and Annexin V staining was performed to distinguish apoptosis from necrosis. Only the Annexin V-positive population was recognized as apoptotic cells. In some experiments, BHK-21 cells were transfected with bcl-2–IRES–DsRed, D34A–IRES–DsRed or control DsRed. Alternatively, cells were co-transfected with the given genes plus the reporter plasmid pEGFP-C1 (Clontech) at a DNA concentration ratio of 5:1 and then infected with JEV (m.o.i. of 1) at 12 h post-transfection. Apoptotic cells with green fluorescence were analysed by flow cytometry. In some experiments, the infected, GFP-positive, rounded-up BHK-21 and N18 cells were examined microscopically for the apoptotic hallmarks of cell shrinkage and nuclear condensation (Yu et al., 2002) at the indicated times post-infection (p.i.).

**Caspase activity assay.** JEV-infected cells (2 × 10⁶) were lysed at various times p.i. and the supernatants were incubated with fluorescence caspase substrate (Clontech): 1 mM Ac-DEVD-fac for
caspase-3, 2 mM Ac-IETD-fac for caspase-8 or Ac-LEHD-fac for caspase-9. Results were obtained using a spectrophotometer (TopCount; Tecan) with excitation at 400 nm and absorption at 505 nm.

RESULTS

Caspase activation and Cyto-c translocation during JEV-induced apoptosis

We have established previously that JEV infection can trigger a typical apoptotic cell death from different types of target cell (Liao et al., 1997). Here, we further investigated how caspase activation occurs in JEV-infected cells. Using fluorescent peptide substrates containing individual caspase-specific cleavage sites, we found that, in neuronal N18 cells, caspase-3, -8 and -9 were first activated after JEV infection at 24 h p.i. and peaked at 36 h p.i. (Fig. 1a). The activation of caspase-9 suggested that JEV infection may disturb the homeostasis of mitochondria, thereby causing the target cells to undergo mitochondrion-dependent apoptosis. To test this possibility, we infected N18 cells with JEV at an m.o.i. of 5 and collected the cytosolic fraction of infected cells to determine the release of Cyto-c from mitochondria. Western blot analysis showed that cytosolic Cyto-c was detected as early as 18 h p.i. in JEV-infected N18 cells (Fig. 1b) and that the levels of Cyto-c release increased gradually, corresponding well to the severity of CPE and the number of dead cells seen following JEV infection (data not shown). When we compared the kinetic changes in the amounts of cytosolic Cyto-c with those of p17, the cleavage product and active form of caspase-3 (CPP32), we found that Cyto-c release appeared to be one of the upstream apoptotic signals for caspase-3 activation during productive JEV replication (Fig. 1c). Similar results for the translocation of Cyto-c and caspase activation (Lee et al., 2005) could also be observed in JEV-infected BHK-21 and NT-2 cells (data not shown). To demonstrate that JEV replication was the direct cause of caspase activation, JEV-infected BHK-21 cells were treated with ribavirin, a specific RNA-dependent RNA polymerase inhibitor. Ribavirin appeared to inhibit JEV replication (Fig. 2a), to enhance cell-survival rates (Fig. 2b) and to block viral protein expression (Fig. 2d), as well as concurrently suppressing virus-induced caspase-3 activation in a dose-dependent manner (Fig. 2c).

![Fig. 1. JEV infection triggers Cyto-c release and caspase activation. (a) Caspase activation was analysed using fluorescent peptide substrates (see Methods). Total cell lysates from JEV-infected N18 cells at the indicated times were incubated with caspase peptide substrates and the amount of cleaved product was measured using a fluorimeter. Results are shown as means ± SD. (b) Western blot of Cyto-c release and caspase-3 activation after JEV infection. M, Mock infection. p32 indicates the intact CPP32/caspase-3, whilst p17 corresponds to its active cleaved product. Full-length PARP (p117) and its cleavage product (p85) were also detected. (c) The relative photodensities of the protein bands of Cyto-c and cleavage product p17 of CPP32 on the Western blot in (b) were measured using a bioimaging analyser system (BAS-1000; Fuji Film) and their band densities at 36 h p.i. were arbitrarily set at 100 %. Virus titres in the supernatant of JEV-infected N18 cells are shown at the indicated times p.i.](image-url)
CsA and RR inhibit JEV-induced apoptosis

Given that JEV infection triggers damage of mitochondria, translocation of Cyto-c and activation of downstream caspases, we further explored whether increased outer mitochondrial membrane permeability was essential for JEV-induced apoptosis. CsA has been shown to prevent the early decrease in mitochondrial membrane potential ($\Delta \Psi_m$), thereby blocking cell death in several systems by stabilizing the PT pores (Halestrap et al., 2002). We found that, in CsA-treated N18 (Fig. 3a) and BHK-21 (data not shown) cells, 61 ± 9 and 31 ± 6% of cells, respectively, could be rescued 3.5 days after JEV infection at an m.o.i. of 5, whereas in untreated N18 (Fig. 3a) and BHK-21 (data not shown) cells, the survival rate was 18 ± 7% and <1%, respectively. In addition, the PT pores of mitochondria were also affected due to the massive Ca$^{2+}$ influx into mitochondria from the cytosol. Pharmacological block of the Ca$^{2+}$ influx by RR has been also shown to achieve inhibition of $\Delta \Psi_m$ and thus stabilizes the PT pores (Kessel et al., 2005). In JEV-infected N18 cells (m.o.i. of 5), we found that 10 μM RR could significantly rescue the cell survival rate from 66 ± 8.2 to 95 ± 8% at day 2 p.i. (Fig. 3b). When examining the role of mitochondria in the JEV-mediated cell-death process, we found that the dose of 10 μM CsA or RR used did not have a cytotoxic effect on the target cells and did not influence virus titres (data not shown). In contrast, higher CsA concentrations of >20 μM, although conferring more cellular protection, caused an approximate 100-fold reduction in JEV titres in BHK-21 and N18 cells. We also observed that, when compared with solvent controls, treatment of JEV-infected N18 cells with 10 μM CsA not only inhibited the dissociation of Cyto-c from mitochondria (Fig. 3c) but also significantly attenuated the activation of caspase-9 and -3, but not that of caspase-8 (Fig. 3d). These results...
suggested that stabilizing mitochondrial PT pores leads JEV-infected cells to tolerate the mitochondrial death signal, probably involving blockage of the massive Ca\(^{2+}\) influx from the cytosol into the mitochondria.

**Caspase-resistant bcl-2 is more potent than wild-type bcl-2 in protecting JEV-infected cells from apoptosis**

Overexpression of bcl-2 has been found to delay the JEV-induced apoptotic process and thereby cause persistent JEV infections in infected BHK-21 cells that would otherwise have been killed (Liao et al., 1997, 1998). The protective effect of Bcl-2 in JEV-infected cells also supports the suggestion that mitochondria are involved in JEV-induced apoptosis. However, in a previous study (Liao et al., 1998), only approximately 5–10% of bcl-2-overexpressing BHK-21 cells (B2-5 cells) survived after 3 days of JEV infection, and the cleaved fragment of Bcl-2 was readily observed in infected cells. It has been demonstrated that activated caspase-3 can proteolytically cleave Bcl-2, and, instead, the resulting pro-apoptotic fragmental Bcl-2 further promotes apoptotic processes in the stressed cells (Cheng et al., 1997; Grandgirard et al., 1998). To examine whether the cleavage of Bcl-2 may play a role in the apoptosis caused by JEV infection, we transiently transfected a caspase-3-resistant human bcl-2 gene (carrying the D34A mutation) into BHK-21 cells and infected the transfectants with JEV to examine their apoptotic response (Grandgirard et al., 1998). As shown in Fig. 4, we found that transient expression of caspase-resistant D34A indeed inhibited JEV-induced apoptosis more potently than wild-type Bcl-2 in infected cells, as shown by analysis of apoptotic morphology (Fig. 4a) and flow cytometry (Fig. 4b). These results illustrated that caspase activation and Bcl-2 play crucial roles in determining the cell’s fate when responding to virus attacks.

**Artificial caspase inhibitors block JEV-induced apoptosis without modulating virus titres**

To examine further the relationship between the caspase activation and JEV-induced apoptosis, artificial cell-permeable caspase inhibitors were utilized in the following experiments. Ac-YVAD-fmk and Ac-DEVD-fmk are classified as caspase-1- and caspase-3-specific inhibitors, respectively (Villa et al., 1997). Ac-YVAD-fmk, a pan-caspase inhibitor, blocks activation of caspase-1, -3, -6 and -7 (Villa et al., 1997). Addition of Ac-YVAD-fmk or Ac-DEVD-fmk showed a dose-dependent pattern of rescue from JEV-induced cell death (Fig. 5a), and Western blot analysis further confirmed that the addition of 20 \(\mu\)M Ac-
DEVD-fmk effectively blocked degradation of full-length CPP32 and PARP in JEV-infected cells (Fig. 5b). We found that, despite these peptide caspase inhibitors having different target specificities, their suppression of JEV-induced cell death appeared to vary only slightly (Fig. 5c). Notably, JEV-infected cells treated with artificial caspase inhibitors did not interfere with virus yields at any of the time points examined (Fig. 5d), suggesting that, although these inhibitors delay the JEV-triggered apoptotic processing, completion of the JEV life cycle does not require caspase activation. Nevertheless, these synthetic inhibitors failed to induce persistent JEV infection when the cultured period was extended (data not shown).

Viral proteins p35 and CrmA and cellular FLIP attenuate JEV-induced apoptosis

The baculovirus p35 protein and poxvirus CrmA protein have been used as virus caspase inhibitors in several apoptotic models. Overexpression of p35 blocks activation of caspase-1 and -3, whilst CrmA inhibits the activity of caspase-1 and -8 in apoptotic cells (Dorstyn & Kumar, 1997; Tschopp et al., 1998). We investigated the effect of p35 or CrmA on JEV-induced apoptosis in N18 and BHK-21 cells. We found that both p35 (Fig. 6a, d) and CrmA (Fig. 6b) showed a comparable block in JEV-induced apoptosis in a dose-dependent manner in infected N18 or BHK-21 cells. Quantitatively, p35 seemed to provide a slightly more potent anti-apoptotic effect than CrmA following JEV-induced apoptosis (Fig. 6a, b), similar to previous observations for reactive oxygen species (ROS)-induced apoptosis (Datta et al., 1997). In addition, we noticed that stable p35 expression helped JEV to establish a persistent infection in the target cells (Fig. 6f), suggesting that, in addition to being a well-known caspase inhibitor, p35 may also confer an additional as-yet-unidentified anti-apoptotic function to rescue doomed JEV-infected cells.

To characterize further the role of caspase-8 in JEV-infected cells, a cellular version of the caspase-8 inhibitor, c-FLIP, was transiently overexpressed in N18 cells. Higher heterodimer affinity between c-FLIP and procaspase-8 inhibits caspase-8 activation by sequestering the dormant procaspase-8 from the DISC formation (Peter, 2004). We found that overexpression of c-FLIP in N18 cells indeed enhanced the cell survival rates in response to JEV infection (Fig. 6c). Similarly, the enforced expression of p35 (Fig. 6e), CrmA or c-FLIP (data not shown) did not significantly affect virus titres, at least during primary JEV infection. Taking these results together, despite not affecting virus titres, inhibition of apoptotic responses by both artificial and natural caspase inhibitors established the crucial role
of caspase activation in the process of JEV-induced cell death.

**JEV-induced apoptosis does not require caspase-3 activation in MCF-7 cells**

Given that JEV-induced apoptosis can be mediated by caspase-8 activation, we further explored the connection between the activation cascade of the mitochondrial/caspase-9 and FADD/caspase-8 pathways. Oligomerization of the death receptors or activation of the downstream caspase-3 can promote the autocleavage of procaspase-8 (Muzio et al., 1998; Slee et al., 1999). In JEV-infected cells, the activation of caspase-8 is concomitant with that of caspase-3 (see Fig. 1a). To elucidate the hierarchy of initiator caspases and to eliminate the interfering influence from the downstream effector caspase-3, a mammary cancer cell line, MCF-7, with no caspase-3 activity (Yang et al., 2001) was used to study JEV infection. As shown in Fig. 7(a) by a virus one-step growth curve, MCF-7 cells appeared to support productive JEV replication, with titres peaking at around 40 h p.i. Apparent apoptosis (55.4±4.8 %) induced by JEV could be detected in infected MCF-7 cells at 60 h p.i. by flow cytometry using Annexin V plus propidium iodide staining (Fig. 7b). This JEV-induced apoptosis in MCF-7 cells also triggered the gradual release of Cyto-c from mitochondria, beginning at 24 h p.i. (Fig. 7c). Caspase-9 was activated at 36 h p.i. and downstream caspase-6 was activated at 36 h p.i.; another apical caspase-8 was also activated at 24 h p.i. (Fig. 7d). These results indicated that JEV does not require caspase-3 activity to induce apoptosis in MCF-7 cells. As both apical caspase-8 and -9 were activated, JEV appeared to simultaneously trigger the death receptor and the mitochondrial pathways in parallel in the caspase-3-deficient MCF-7 cells.
Caspase-8 activation in JEV-induced apoptosis is not mediated through an FADD-dependent pathway

To clarify whether the death signal to trigger caspase-8 activation was extrinsic during JEV-induced apoptotic processing, we stably expressed a FADD-DN gene (Chen & Lai, 2001; Newton et al., 2001) in MCF-7 and BHK-21 cells (Fig. 8a), in which FADD-DN can interrupt the external Fas/TNFR sending apoptotic signals inside the cell to trigger the internal caspase-8 activation. We found that FADD-DN expression in MCF-7 clones, despite successfully reducing TNF-α-induced apoptosis, failed to block JEV-induced apoptosis (Fig. 8b); similar results were also seen for BHK-21 cells expressing FADD-DN (data not shown). Treatment of JEV-infected MCF-7 cells with 50 or 100 μM z-IETD-fmk (a caspase-8-specific inhibitor) significantly enhanced the survival rate compared with the untreated control (Fig. 8c), indicating that caspase-8 plays a role, at least in part, in JEV-induced apoptosis in MCF-7 cells. In addition, FADD-DN expression did not appear to interfere with activation of caspase-9, -8 or -3 in either JEV-infected BHK-21 (Fig. 8d) or MCF-7 cells (Fig. 8e).

Taken together, these observations suggest that the initiator caspase-8 activation induced by JEV infection may be mediated through the death-receptor pathway in an FADD-independent and as-yet-unknown manner.

DISCUSSION

Although JEV infection appears to cause both apoptosis and necrosis of target cells, we failed to distinguish the hierarchy between the apoptotic and necrotic pathways following JEV infection in this study. The interplay between the two processes is of interest and remains to be studied further. Despite the exact upstream death signals remaining unclear, the results from infected neural N18 (Fig. 1) and fibroblast BHK-21 (Fig. 4) cells, together with our previous findings (Liao et al., 1997), clearly indicate that JEV is not only an inducer of apoptotic death but can also activate caspase cascades in infected cells (Figs 1, 7 and 8). The simultaneous activation of caspase-8 and -9 (Fig. 7) together with the specific suppression of caspase-9, but not caspase-8, by CsA (Fig. 3d) seem to suggest that JEV can trigger the death-receptor pathway and the mitochondrial...
pathway separately and in parallel. It will be of interest to reveal the upstream signals that trigger these two pathways following JEV infection.

Virus replication can cause mitochondrial injury by the generation of secondary toxic metabolites, such as ROS, nitric oxide and superphysiological Ca\(^{2+}\) concentrations, which may serve as the upstream signals to modulate the mitochondrial PT pores, leading progressively to \(\Delta \Psi_m\) collapse, Cyto-c release and caspase-9 activation (Cai & Jones, 1998; Green & Kroemer, 2004; Tan et al., 1998). In an early stage of JEV-induced apoptosis, we found a change in mitochondrial potential and the generation of ROS in N18 cells (Lin et al., 2004), and even cells treated with UV-inactivated JEV caused ROS-dependent mitochondrial injury and a decrease in \(\Delta \Psi_m\) (Lin et al., 2004). Alternatively, we have shown previously that a high level of JEV replication on the endoplasmic reticulum (ER) membrane triggers classical ER stress and an unfolding protein response (Su et al., 2002), which may lead to depletion of the storage of Ca\(^{2+}\) in the ER lumen, causing an elevation of Ca\(^{2+}\) concentrations to abnormal levels and activating ER-associated caspase-12, which in turn triggers the mitochondrial pathway. It was therefore not unexpected to see in this study that stabilizing the mitochondrial PT pores by CsA or blocking the influx of Ca\(^{2+}\) into the mitochondria by RR could delay the progression of JEV-induced apoptosis. The ectodomain of the M protein of JEV has been shown to be an apoptotic inducer (Catteau et al., 2003a), probably by triggering \(\Delta \Psi_m\) collapse, but not by generating ROS or activating caspase-9 (Catteau et al., 2003b). Conceivably, JEV infection may also cause disruption of \(\Delta \Psi_m\) by producing certain toxic secondary metabolites, such as ROS, nitric oxide or abnormal Ca\(^{2+}\) concentrations in the target cells.

Several RNA viruses have been shown to readily activate the caspase-8-associated apoptotic pathway (Bitzer et al., 1999; Clarke & Tyler, 2003; Liu et al., 2006). In the case of reovirus, the induced apoptosis involves the activation of death receptors DR5 or DR4 with TNF-related apoptosis-inducing ligand (TRAIL, also called Apo2L), which depends mainly on the intracellular FADD association with procaspase-8 (Clarke & Tyler, 2003). On the other hand, although caspase-8 is often activated through the death-receptor pathway in many systems, Sendai virus can activate apical caspase-8 without involvement of the upstream death receptors during the apoptotic process (Bitzer et al., 1999), but whether this caspase-8 activation is dependent or not on association with FADD to form DISC remains unclear. Our

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**Fig. 7.** JEV infection triggers apoptosis and caspase activation in the absence of caspase-3 in MCF-7 cells. (a) JEV replication levels were determined in MCF-7 cells by a virus one-step growth curve. (b) Apoptotic cells in MCF-7 cells infected by JEV (m.o.i. of 1) were analysed by flow cytometry using Annexin V staining at the indicated times p.i. M, Mock infection at 60 h p.i. (c) Cyto-c release in the cytosolic fraction was detected by Western blotting of JEV-infected MCF-7 cells at the times indicated p.i. (d) Activation of different caspases after JEV infection was determined using specific fluorescent peptide substrates (see Methods) at the indicated times p.i. Results are shown as means ± SD derived from three independent experiments. *, \(P<0.05\).
results provide a similar example in that JEV infection was shown to induce apical caspase-8 activation in a FADD-independent manner, leading to apoptosis, even without caspase-3 (Figs 7 and 8). Suppression of the mitochondrial apoptotic pathway by CsA treatment failed to inhibit JEV-induced caspase-8 activation (Fig. 7), indicating that effector caspase-3 is not always essential for the caspase-8-mediated apoptotic pathway in JEV-infected cells. Using the same FADD-DN approach as we did in this study, it has been shown previously (Schnantz et al., 2001) that transforming growth factor-β induces FADD-independent activation of caspase-8 in the apoptotic pathway regulated by p38 MAP kinase. In our previous study (Su et al., 2002), we demonstrated that JEV replication triggers the unfolding protein response in infected cells as indicated by induction of certain chaperones, resulting in apoptotic cell death. Moreover, JEV infection also activates the expression of transcription factor CHOP/GADD153 and triggers the activation of p38 MAP kinase. Ectopic expression of CHOP could enhance JEV-induced apoptosis, whereas treatment with a p38-specific inhibitor, SB203580, appeared to attenuate JEV-induced apoptosis. Conceivably, it could be the activated p38 MAP kinase that triggers FADD-independent activation of caspase-8 in JEV-induced apoptosis. Alternatively, the possibility that the TRAIL pathway might also participate in JEV-induced cell death cannot be excluded, as the cytokine TRAIL has previously been reported to activate the caspase cascade by an FADD-independent mechanism (Pan et al., 1997; Sheridan et al., 1997).

In summary, we found that JEV replication induced a caspase-mediated apoptosis and that caspase inhibitors, although able to alleviate the apoptotic process, had no effect on virus replication, consistent with a previous observation from West Nile virus-induced apoptosis (Kleinschmidt et al., 2007). These observations therefore suggest that flavivirus replication does not require caspase activation to complete the virus life cycle. JEV infection not only triggers caspase-9 activation via the mitochondrial pathway but also induces caspase-8 activation separately through a FADD-independent death-receptor pathway. Taken together, our results suggest that caspase cascades triggered by JEV infection may start from activation of the apical caspase-8 and -9, probably through a FADD-independent but mitochondrial-dependent pathway.

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Apoptosis leads to a FADD-dependent but Fas ligand-independent cell death in
modulated by Bcl-XL and caspase inhibitors. 

Rubella virus-induced apoptosis varies among cell lines and is
modulated by Bcl-XL and caspase inhibitors. 

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