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Semliki Forest Virus-Induced Endoplasmic Reticulum Stress Accelerates Apoptotic Death of Mammalian Cells[▽][†]

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The alphavirus Semliki Forest virus (SFV) and its derived vectors induce apoptosis in mammalian cells. Here, we show that apoptosis is associated with the loss of mitochondrial membrane potential followed by the activation of caspase-3, caspase-8, and caspase-9. Cell death can be partially suppressed by treatment with the pan-caspase inhibitor zVAD-fmk. To determine the role of SFV structural proteins in cell death, the temporal course of cell death was compared in cells infected with SFV and cells infected with SFV virus replicon particles (VRPs) lacking some or all of the virus structural genes. In the absence of virus structural proteins, cell death was delayed. The endoplasmic reticulum (ER) stress response, as determined by the splicing of X-box binding protein 1 (XBP1) transcripts and the activation of caspase-12, was activated in virus-infected cells but not in VRP (SFV lacking structural genes)-infected cells. The C/EBP-homologous protein (CHOP) was upregulated by both virus and VRP infections. The virus envelope proteins but not the virus capsid protein triggered ER stress. These results demonstrate that in NIH 3T3 cells, SFV envelope glycoproteins trigger the unfolded protein response of the ER and accelerate apoptotic cell death initiated by virus replicase activity.

Apoptosis can be triggered by many external stimuli, including virus infections. Whereas the apoptosis of virus-infected cells may facilitate or even be required for the release of some viruses, this process generally is perceived as an altruistic antiviral response that limits virus release and the infection of surrounding cells (1, 58). The effectiveness of apoptosis as an antiviral response is underlined by the existence of the many antiapoptotic strategies employed by viruses (10, 89). Most viral antiapoptotic responses to date have been observed in DNA viruses. Many RNA viruses have relatively short replication times and replicate to high titers before the infected cell undergoes cell death. These viruses may not need to suppress apoptosis. For many RNA viruses, the mechanism, or more likely mechanisms, by which cells detect virus infection and initiate cell death, the temporal course of events, and whether the virus interferes with these remain to be determined.

Alphavirus infections, including Semliki Forest virus (SFV) and Sindbis virus (SINV), have been at the forefront of studies of virus-induced cell death and provide important tractable model systems to study many aspects of virus pathogenesis (2, 17, 18, 31). Alphaviruses enter cells by receptor-mediated endocytosis (33). Fusion occurs in endolysosomes (53). Virus RNA replication and transcription occur in specialized invaginations on smooth membrane vesicles in the cytoplasm (23, 43). The viral genome has a methylated nucleotide cap at the 5′ end, is polyadenylated at the 3′ end, and is translated like cellular mRNA. The genome is split into two distinct open

reading frames (ORF) (see Fig. 4). The 5′ two-thirds of the genome encodes the nonstructural protein (nsP) ORF, which codes for nsP-1, nsP-2, nsP-3, and nsP-4; together these form the virus replicase that replicates and transcribes virus RNA. The 3′ one-third of the genome encodes the structural protein ORF that codes for the capsid (C), 6K, and envelope glycoproteins (E1, E2, and E3) (40). The transcription of new positive-strand genomic RNA starts at the 3′ end of the genome-complementary negative strand. Late in infection, a subgenomic mRNA is generated by the initiation of transcription at an alternative, subgenomic promoter located 3′ of the structural genes on the negative strand. This subgenomic RNA is translated into the virus structural proteins. The virus envelope glycoproteins, as with cellular glycoproteins, are processed through the endoplasmic reticulum (ER) and Golgi complex (40, 41). New virions are assembled at and bud from the plasma membrane (76, 77).

Virus replicon particles (VRPs) can be generated from many alphaviruses, including SFV (22, 74). VRPs are structurally the same as virus, except that the encapsidated genome lacks all or part of the structural ORF (see Fig. 2A). Upon the infection of a cell, VRPs replicate their genome but cannot make new virus particles. The structural ORF can be replaced by a foreign gene, for example, enhanced green fluorescent protein (eGFP). Recombinant replicating alphavirus vectors and VRPs are in development as antitumor agents and vaccines (5, 19, 81, 82).

Alphavirus infections of mammalian cells, including SFV, SINV, Venezuelan equine encephalitis virus, and Ross River virus, trigger apoptosis (3, 28, 35, 47, 71). Exactly how alphavirus apoptosis is triggered remains unclear. SINV-induced apoptosis does not appear to require virus replication; the entry of UV-inactivated virus into a cell can trigger ceramide release and cell death (36, 37). In contrast, UV-inactivated

SFV does not kill cells (86). SFV genome replication without structural protein expression is sufficient to kill cells (29, 86). A number of alphavirus mutants with reduced cytopathogenicity have mutations in nsP2, and the expression of nsP2 alone is cytotoxic (15, 20, 21, 25, 26, 30, 63, 80). The expression of the transmembrane domains of the SINV glycoproteins also has been shown to result in cell death (39). SINV-induced apoptosis is mediated by Bad, and SFV-induced apoptosis is mediated by Bak (57). The apoptosis of both SINV- and SFV-infected cells can be delayed by the overexpression of Bcl-2 or Bcl-xl, resulting in persistently infected cultures (11, 57, 59, 71, 86). The inhibition of caspase-8 delays SINV-induced apoptosis, and cells deficient in caspase-9 have delayed apoptosis following SFV infection (62, 86). Alphavirus-induced apoptosis has been linked to cell proliferation. The inhibition of the cell cycle protein Ras leads to delayed cell death after SINV infection (38). Neither p53 nor p53 upregulated modulator of apoptosis (PUMA) is required for SFV-induced cell death (29, 86).

The possibility that structural proteins trigger the unfolded protein response (UPR) of the ER and that this contributes to cell death has not been investigated for alphaviruses. The UPR is a cellular response to the overaccumulation of unfolded proteins in the lumen of the ER (69). This response is important in the physiology of healthy cells and functions to downregulate protein synthesis in response to unfolded protein buildup (69). The buildup of unfolded proteins is monitored by many ER protein chaperones, including immunoglobulin-binding protein (BiP). BiP is located in the lumen of the ER and in unstressed cells binds to the luminal domains of three ER membrane bound proteins: eukaryotic translation initiation factor 2-alpha kinase 3 (PERK), inositol-requiring protein-1 (IRE1), and activating transcription factor-6 (ATF6). The activation of these proteins triggers a series of events that attempt to restore normal function by pausing translation and increasing protein folding capacity. If this fails, apoptosis can be triggered by, for example, the induction of the proapoptotic C/EBP-homologous protein (CHOP) or by calcium-dependent mitochondrial membrane permeabilization (7, 65, 72).

The aim of this study was to identify whether the SFV-induced cell death of NIH 3T3 mouse fibroblasts involves the extrinsic or intrinsic pathway of apoptosis, as well as to investigate whether an ER stress response is activated and whether this contributes to cell death. We show that SFV-infected cells have an early loss of mitochondrial membrane integrity followed by the activation of caspase-3, caspase-8, and caspase-9, which is indicative of the activation of the intrinsic pathway of apoptosis. XBP-1 mRNA is spliced and caspase-12 activated following infection with SFV but not following infection with VRPs (lacking structural genes); CHOP, in contrast, is activated by both virus and VRP infection. By comparing the infection of virus and VRPs lacking some or all virus structural protein-coding sequences, we demonstrate that virus envelope proteins, but not virus capsid protein, initiate the ER stress response and accelerate apoptotic cell death.

MATERIALS AND METHODS

Virus and replicons. Infectious SFV4 virus was generated from the pSP6-SFV4 plasmid by following the method of Liljestrom et al. (51). Virus was purified through a sucrose cushion (18). EGFP-expressing VRPs were prepared using pSFV1-d1eGFP (SFV replicon vector containing the nonstructural ORF and a d1eGFP gene under the control of the subgenomic promoter) and SFV pHelper1 (containing the structural ORF) (18, 50). D1eGFP is a short-half-life form of eGFP (48). A VRP, designated RC, was constructed to express wild-type capsid protein from the subgenomic region. A second VRP, designated RE, was designed to express envelope glycoproteins but no capsid protein. Both were made using the replicon plasmid pSFV1. In RE the subgenomic promoter region is followed by 102 nucleotides (34 amino acids [aa]) from the SFV capsid region so as to include the translation enhancer element, followed by the foot-and-mouth disease virus autoprotease 2A sequence. This generates wild-type expression levels of virus envelope proteins. VRPs were generated in cells cotransfected with helper RNAs encoding the missing structural genes as described for the SFV two-helper system (74).

Cells. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) with 10% newborn calf serum (Invitrogen, United Kingdom) and were used in all experiments. Cells always were infected with SFV4 or VRPs at an MOI of 30 (this MOI was shown by immunostaining to infect 95 to 100% of all NIH 3T3 cells).

XBP1 analysis. At 7, 9, 11, and/or 13 h postinfection, RNA was isolated from infected cells using a Qiagen RNeasy kit. The RNA then was reverse transcribed and amplified by PCR with primers specific for XBP1. Primers used were the following: forward, 5′-AAACAGAGTAGCAGCGCAGACTGC-3′; reverse, 5′-GGATCTCTAAAACTAGAGGCTTGGTG-3′. The PCR product then was treated with the restriction enzyme PstI (Promega) and run on an agarose gel. As a positive control, tunicamycin was used to induce ER stress by inhibiting the glycosylation of proteins (16, 83).

Assay of caspase activity. Cells were seeded in 96-well plates at 1×10^4 cells per well, grown overnight, and infected with virus or VRPs. At various times postinfection, cells were lysed and assayed for caspase activity. Caspase-glo-3/7, caspase-glo-8, and caspase-glo-9 (Promega) assay kits were used according to the manufacturer's instructions. Assays were carried out in quadruplicate and repeated three times. Negative controls were mock-infected cells. Antibodies for cleaved caspase-12, β-actin (Cell Signaling) and CHOP (Santa Cruz), were used in immunoblot analyses.

Measuring MMP. JC-1 powder (Sigma) was dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml, aliquoted, and frozen at −20°C. A six-well plate, with 20- by 20-mm coverslips in all wells, was seeded with 3×10^5 cells per well and incubated for 16 h at 37°C. Cells were infected or mock infected, and at various times postinfection, medium (1 ml) containing 100 ng/ml JC-1 was added to each well, and the plates were incubated for 1 h before visualization by fluorescence microscopy. As a positive control (loss of mitochondrial membrane potential [MMP]), cells were treated with 4% neutral buffered formaldehyde for 1 h. Green (loss of MMP) and orange cells (intact MMP) were quantified by counting cells in 15 different fields and determining the averages.

Cell viability assay. Cell viability was determined using the Wst-1 reagent (Roche) according to the manufacturer's instructions. Wst-1 is a tetrazolium salt that is cleaved to a formazan dye by the active mitochondrial dehydrogenases of live cells. Levels of dye are determined spectrophotometrically at 420 to 480 nm. As cells lose viability, less formazan dye is made. Cells were seeded at 3×10^4 per well in 96-well plates and incubated overnight. At the required time, 10 μl of Wst-1 was added to each well (containing 100 μl medium). Spectrophotometric readings were taken after 1 h using a microplate reader. Replicates of four wells of cells were used for each time point. Experiments were repeated at least three times.

Knockdown of caspase-12. NIH 3T3 cells were transfected with small interfering RNA (siRNA) (against caspase-12 or scrambled mock) (silencer select siRNA; Ambion) using dharmafect 1 (Thermo Scientific) and incubated for 48 h before RNA or protein was isolated from the cells. The RNA was converted to cDNA, and a fragment of the caspase-12 transcript was amplified. Total protein content was probed for caspase-12 by immunoblotting using an anti-caspase-12 antibody (Cell Signaling).

Caspase inhibition. A pan-caspase inhibitor, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (zVAD-fmk or zVAD; Promega), was used to inhibit caspase activity. The optimum concentration of zVAD was determined to be 50 to 200 μM. zVAD was mixed with cell culture media and added to cells 2 h before apoptotic stimulus. The apoptotic stimulus (e.g., SFV) then was added, and the cells again were incubated with cell culture medium containing zVAD (200 μM). Cell viability was measured by Wst-1 assay.

RESULTS

SFV4-infected cells have a loss of MMP, and SFV-induced cell death is reduced but not completely suppressed by treatment of infected cells with a pan-caspase inhibitor. The extrinsic pathway of apoptosis is initiated by the ligation of cell death receptors and includes the early activation of caspase-8. The intrinsic pathway involves the loss of mitochondrial membrane potential (MMP), the release of cytochrome *c*, and the early activation of caspase-9. Both pathways activate downstream caspases, including caspase-3 (52). To investigate MMP in SFV-infected cells, the cationic dye JC-1 was used (68, 75). In mitochondria with an intact MMP, JC-1 aggregates and fluoresces orange. The loss of MMP results in the dissociation of these aggregates, and the JC-1 monomers fluoresce green. Parallel cultures of NIH 3T3 cells were infected (MOI of 30) with SFV4. This MOI resulted in the synchronous infection of all cells in the culture (data not shown). Cells then were treated with JC-1, and the percentage of green or orange cells was determined at various times postinfection (Fig. 1A). Early in infection (6 h) most cells had intact MMP (orange). MMP disruption was apparent in >50% of cells by 8 h and in most cells by 13 h.

We next investigated whether SFV-induced cell death in NIH 3T3 cells could be blocked by the pan-caspase inhibitor zVAD-fmk (zVAD). Replicate cultures were incubated with zVAD (200 μM) for 2 h and then infected with SFV4 in medium containing the same concentration of zVAD. Caspase-3/7 activity was assayed in zVAD-treated cultures and was below detectable levels. The viability of the cultures was measured at 20 and 30 h postinfection and compared to that of uninfected or non-zVAD-treated cultures (30-h time point is shown Fig. 1B). The zVAD-treated, SFV4-infected cultures had a significantly higher viability than the untreated infected cultures at both time points, indicating that zVAD had a protective effect. However, the viability of zVAD-treated cultures infected with SFV4 was significantly lower than that of uninfected cultures at both 20 and 30 h postinfection. This suggests that while caspase-dependent death is important, caspase-independent mechanisms such as the release of apoptosis-inducing factor (AIF) and endonuclease G from mitochondria (12, 87) also play a role in SFV-induced apoptosis. These results are consistent with our previous studies of the effect of zVAD on the SFV-induced apoptosis of neuronal cells in culture (2).

SFV structural proteins contribute to virus-induced cell death. It has been shown previously that SFV vector systems or VRPs trigger cell death, and that the replication of the virus replicon, the replicase gene without the structural protein ORF, is sufficient to trigger cell death (29). How the replication of the replicon initiates apoptosis remains to be determined. It is likely that more than one aspect of virus replication triggers cell death. Virus glycoproteins traffic through the ER and the Golgi complex. The buildup of unfolded proteins in the ER can lead to ER stress and, if this is not resolved, apoptosis. ER stress has been shown to be activated and to upregulate proapoptotic proteins in a number of virus infections (49, 55, 78). To determine whether SFV structural proteins contribute to cell death, rates of cell death were compared in parallel NIH 3T3 cell cultures infected with SFV4 or VRPs at the same MOI. VRPs encapsidating an SFV genome
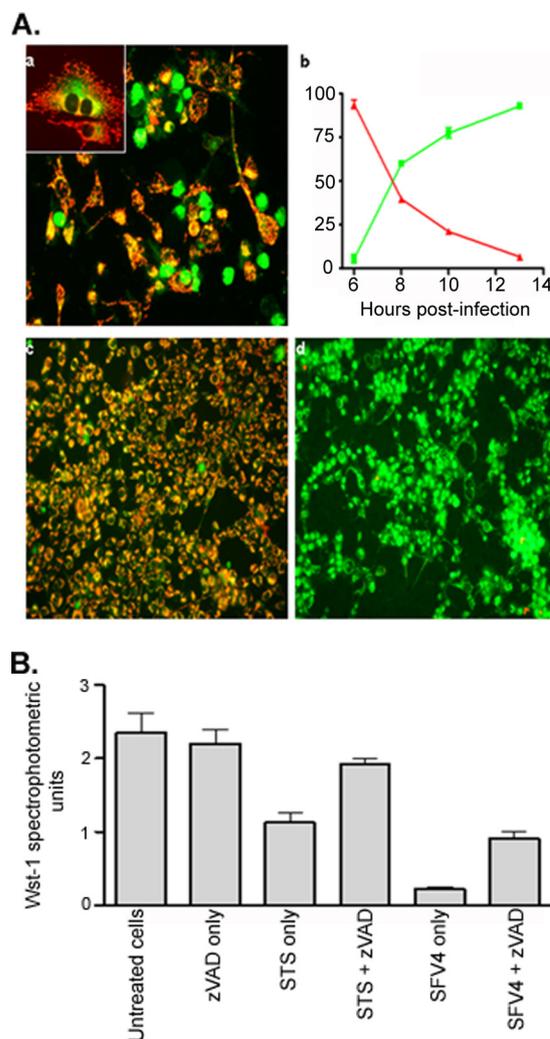

FIG. 1. (A) Loss of MMP in SFV4-infected NIH 3T3 cells as visualized by JC-1 staining. (a) Cells with (orange) and without (green) an intact MMP are easily distinguished using JC-1 staining (SFV4-infected NIH-3T3 cells; MOI = 1.0 at 24 h). Green cells are rounded up and condensed. The inset shows a couple of cells at higher-power magnification (×100). (b) Time course of the loss of MMP following SFV4 infection (MOI, 30); the percentage of green cells (green line) increased, and the percentage of orange cells (red lines) decreased. Each point is the mean count from three replicate cultures in 15 fields selected at random. The experiment was repeated independently twice with similar results. (c) JC-1 staining of uninfected NIH 3T3 cells. (d) JC-1 staining of formaldehyde-treated NIH 3T3 cells. (B) Viability (Wst-1 assay) of NIH 3T3 cells 30 h after infection with SFV4 (MOI, 30) in the presence or absence of zVAD (200 μM), a pan-caspase inhibitor. As controls, cells were treated with staurosporine (STS; 1 μM), a known inducer of apoptosis, for 30 h in the presence or absence of zVAD or were left untreated. The bars represent the means of quadruplicate cultures at each time point. Error bars are standard deviations from the means. This experiment was repeated three times with similar results.

in which the structural ORF was replaced with d1eGFP (Fig. 2A) were used to observe the course of infection in the absence of virus structural protein synthesis and the trafficking of virus glycoproteins through the ER and Golgi complex; eGFP, which does not traffic through the ER and Golgi complex, was included as a convenient marker of infection. The viability of
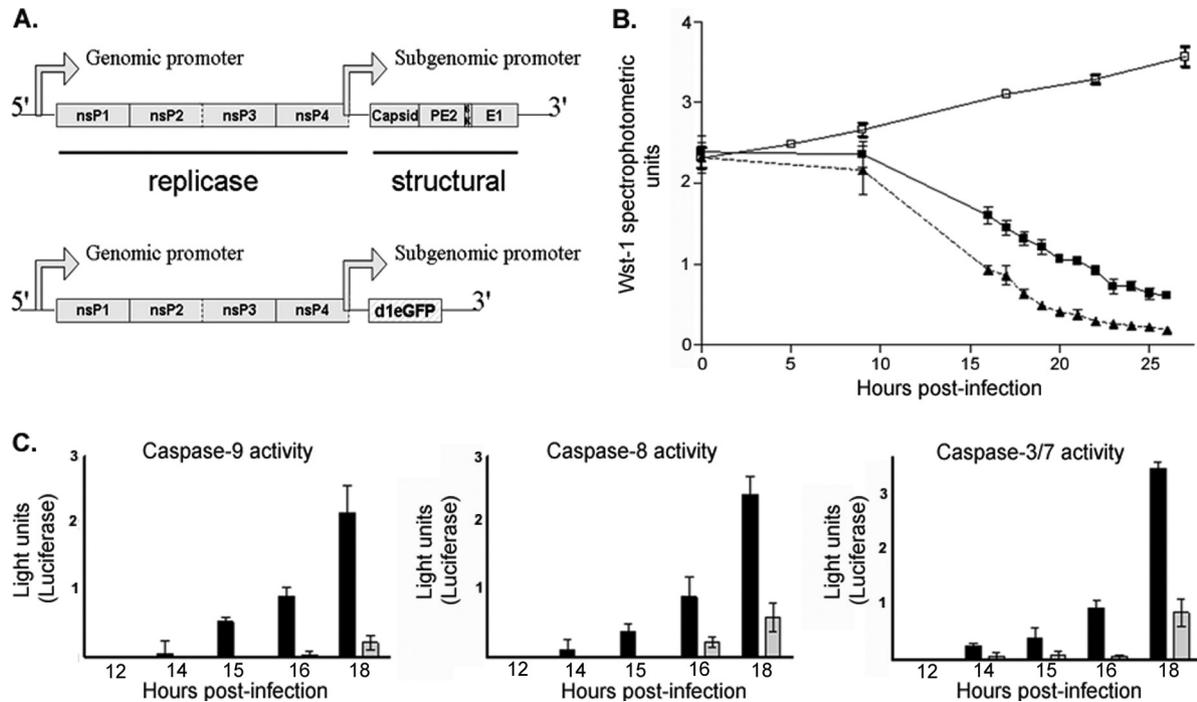
FIG. 2. (A) Representation of the genomes of SFV (upper) and SFV1-d1eGFP VRPs (lower). The replicase ORF comprised of nsP1 to nsP4 is present in both. In addition, the viral genome has the structural ORF comprised of the capsid (C), p62 (precursor of the E2 and E3 envelope glycoproteins), and the E1 envelope glycoprotein. The structural ORF of the VRP genome is replaced with d1eGFP. (B) Viability (Wst-1 assay) of uninfected NIH 3T3 cells (□) and NIH 3T3 cells infected (MOI, 30) with SFV4 (▲) or VRPs (■). Each point is the mean of quadruplicate cultures. Error bars are standard deviations from the means. The experiment was repeated three times with similar results. (C) Replicate cultures of NIH 3T3 cells were infected (MOI, 30) with SFV4 (■) or VRPs (□). Levels of activated caspase-3/7, -8, and -9 were determined at 12, 14, 15, 16, and 18 h postinfection. The bars are means from quadruplicate cultures at each time point. Error bars are standard deviations from the means. This experiment was repeated three times with similar results.

VRP-infected cells was significantly higher during the later stages of infection ($P < 0.05$, Mann-Whitney test) than that of SFV4-infected cells (Fig. 2B), indicating that virus structural proteins contribute to cell death.

Caspase-3/7, -8, and -9 activation is delayed in VRP infection compared to virus infection. To investigate this delay in cell death further, a time course of the activation of caspase-3/7, -8, and -9 in both virus- and VRP-infected NIH 3T3 cells was determined. Replicate cultures were infected with SFV4 or SFV VRPs, and levels of caspase-3/7, -8, and -9 were determined in quadruplicate cultures at 12, 14, 15, 16, and 18 h postinfection (Fig. 2C). No caspase activity was observed at 12 h, but all three caspases were active by 14 to 15 h in virus-infected cells. In VRP-infected cells, however, caspase activation was delayed by approximately 2 h. The loss of MMP preceded caspase activation, and the activity of all three caspases was detected at the same time. These results indicate that in NIH 3T3 cells the intrinsic pathway of apoptosis was activated and are in agreement with a recent study using mouse embryo fibroblasts in which SFV-induced apoptosis was shown to be mediated by Bak and to proceed via the intrinsic pathway with MMP loss, cytochrome *c* release, and the activation of caspase-3, -8, and -9 (86). A delay in caspase activation in VRP infection relative to that of virus infection suggests that the synthesis of virus structural proteins contributes to cell death.

Levels of the ER stress-associated proteins CHOP and activated caspase-12 in SFV- and VRP-infected cells. ER stress

can trigger apoptosis (67) and could be the additional pro-apoptotic signal in virus-infected cells. Prolonged ER stress leads to the activation of caspase-12, elevated levels of CHOP, and apoptosis (54). Normally, CHOP is expressed at very low levels and caspase-12 is present only as inactive procaspase-12. Levels of these two proteins were determined by immunoblot analysis of SFV4- and VRP (lacking all structural genes)-infected cells at 7, 9, 11, and 13 h postinfection (Fig. 3). Uninfected cells and tunicamycin (5 μg/ml)-treated cells were used

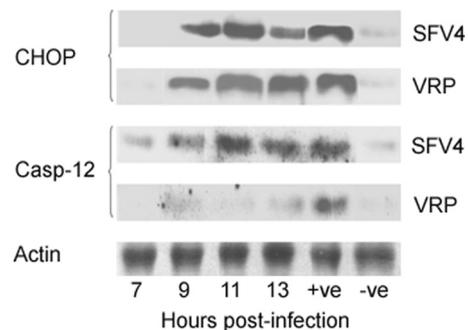
FIG. 3. Levels of CHOP, activated caspase-12, and β-actin proteins as determined by immunoblotting in NIH 3T3 cells infected (MOI, 30) with SFV4 or VRPs. The negative control (-ve) was mock-infected cells. The positive control (+ve) was tunicamycin (5 μg/ml)-treated cells. This experiment was repeated three times with the same result.

as negative and positive controls, respectively, and actin levels were determined as a loading control. CHOP expression was observed as early as 9 h postinfection in both SFV4- and VRP-infected cells. CHOP is upregulated following the phosphorylation of eIF-2α by the ER stress-induced kinase PERK and other kinases, including PKR. We have previously shown PKR to be activated in SFV-infected cells (6). In contrast, activated caspase-12 was observed in SFV4-infected cells starting at 9 h but not in VRP-infected cells, suggesting that virus but not VRPs activate the ER stress response.

Transcripts for X-box binding protein 1 (XBP1) are spliced in SFV- but not in VRP-infected cells. As a second marker of the unfolded protein ER stress response, XBP1 splicing was analyzed. The splicing of XBP1 transcripts is an early indicator of UPR activation (8, 91). Upon the activation of the UPR, IRE1 traffics from the ER to the cytoplasm and splices XBP1 mRNA. Splicing allows the translation of a transcription factor that upregulates, among other genes, multiple chaperones (8, 85, 91). Replicate cultures were infected with SFV4 or VRPs lacking all of the viral structural genes. Infection was confirmed by immunostaining for SFV nsP3 protein in parallel cultures (data not shown). Total RNA was isolated at various times postinfection and reverse transcribed into cDNA. Primers specific for XBP1 then were used to amplify the XBP1 splice variants. The splice variants, and their PCR products, differ by only a few base pairs, which is difficult to see on an agarose gel. The amplified DNA therefore was digested with the restriction enzyme PstI, which cleaves a site present only in the unspliced XBP1 mRNA (8). Spliced XBP1 RNA is not cleaved by PstI and appears as a higher-molecular-weight band. PstI-digested samples derived from SFV4- and VRP-infected cells were run on a gel. PstI-treated samples derived from uninfected cells and from tunicamycin (5 μg/ml)-treated cells served as negative and positive controls, respectively. Following SFV4 infection, levels of spliced XBP1 were background at 7 h but had clearly increased by 9 h (Fig. 4A). In contrast, in VRP- and mock-infected cells, there was no splicing of XBP1 even at 13 h postinfection, confirming the results with caspase-12, that the virus replicon lacking structural genes does not activate the UPR.

SFV envelope proteins but not the capsid protein triggers the ER stress. To determine which of the virus structural proteins, the capsid or envelope glycoproteins, were responsible for activating the UPR in virus-infected cells, VRPs expressing only the capsid (RC) or only the envelope glycoproteins (RE) were constructed (Fig. 4B). Infection with these VRPs again was confirmed by immunostaining for nsP3 in parallel infected cultures. RNA was isolated 13 h postinfection, and the splicing of XBP1 transcripts was assessed (Fig. 4C). Infection with VRPs expressing only the envelope glycoproteins (RE) resulted in the increased splicing of XBP1 transcripts, whereas infection with VRPs expressing only the capsid structural protein (RC) resulted in background levels of XBP1 transcript splicing. Thus, the envelope glycoproteins and not the capsid protein triggered the ER stress response. The experiment was repeated at 24 h postinfection with the same result (data not shown).

Caspase-12 is not required for SFV4-induced mouse fibroblast cell death. SFV4 infection triggered the activation of caspase-12, whereas VRP infection did not. The involvement
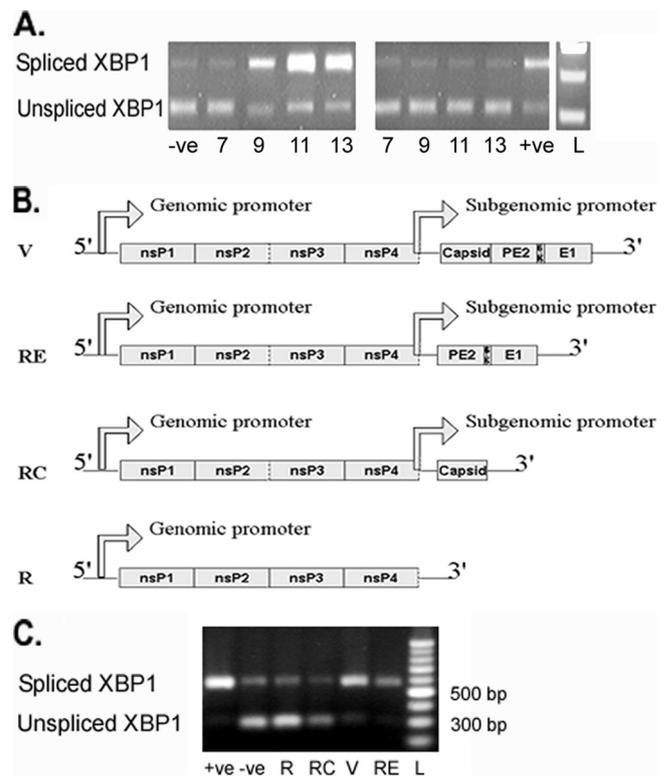

FIG. 4. (A) XBP1 transcript splicing in NIH 3T3 cells infected (MOI, 30) with SFV4 or VRPs. RNA from infected cells was reverse transcribed into cDNA, and XBP1 amplicons were generated by PCR. These were digested with PstI, which only cleaves amplicons derived from unspliced XBP1 transcripts. The digests were run on an agarose gel (shown). The upper (undigested) band represents spliced transcript and the lower (digested) band unspliced transcript. The negative (-ve) control was RNA from mock-infected cells. The positive (+ve) control was RNA from tunicamycin (5 μg/ml)-treated cells. The ladder (L) is a 1-kb DNA ladder (Promega) showing 250-bp (lower), 500-bp (middle), and 750-bp (upper) bands. (B) Genome structure of SFV4 (V), VRPs expressing the nonstructural and envelope proteins only (RE), VRPs expressing the nonstructural and the capsid protein only (RC), or VRPs lacking all structural genes (R) used to infect (MOI, 30) NIH 3T3 cells. (C) Following infection with the virus or VRPs from panel B, XBP1 transcript splicing was assessed (as described above) at 13 h postinfection. The negative control (-ve) was RNA from mock-infected cells. The positive control (+ve) was RNA from tunicamycin (5 μg/ml)-treated cells. The ladder (L) was a 100-bp DNA ladder (New England Biolabs). The experiment was repeated three times with similar results.

of caspase-12 in apoptosis remains controversial, with evidence suggesting it plays an important role in ER stress-induced apoptosis, while other reports suggest it is not required (61, 70). To determine whether caspase-12 activation was responsible for the more rapid cell death observed in virus-infected cells than in VRP-infected cells, rates of cell death were measured in virus-infected cells with a knockdown of caspase-12. NIH 3T3 cells were transfected with siRNAs targeted against caspase-12, incubated for 48 h, and then infected with SFV4 (MOI, 30). The knockdown of caspase-12 was verified both at the transcript level by PCR and at the protein level by immunoblotting. Cell population viability was measured over time using a Wst-1 assay and compared to that of infected control-transfected cells (Fig. 5C). Following SFV infection, cells with

A.

Casp-12
Actin

C-12 siRNA: -ve -ve +ve +ve L

500 bp
400 bp
300 bp
200 bp

B.

Casp-12

Actin
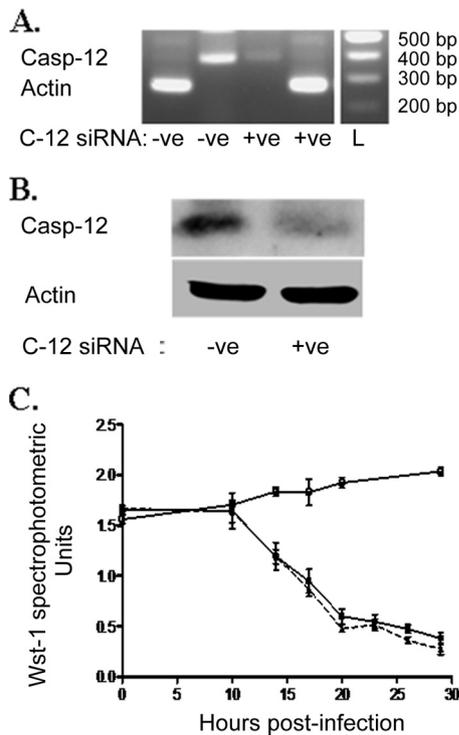
C-12 siRNA :   -ve   +ve

C.

FIG. 5. Rate of cell death was unaffected by a knockdown of caspase-12. (A) NIH 3T3 cells were treated (+ve) with siRNA against caspase-12 (C-12) or with a scrambled control siRNA (-ve). The effects on caspase-12 and, as a control, β-actin transcripts were determined by PCR. (B) Caspase-12 and β-actin protein levels after siRNA treatment. (C) NIH 3T3 cells treated with siRNA against caspase-12 (■) or control siRNA (▲) were infected (MOI, 30) with SFV4, and cell viability (Wst-1 assay) was measured over time. Uninfected cells (□) were included as a control. This was repeated three times with similar results.

a knockdown of caspase-12 died at the same rate as control cells, suggesting that caspase-12 is not required for cell death and is not responsible for the more rapid death of virus-infected than VRP-infected NIH 3T3 cells.

DISCUSSION

The alphavirus infection of mammalian cells generally triggers apoptosis, but the pathways involved remain to be determined. Here, we demonstrate the loss of MMP before the coincident activation of caspase-3, -8, and -9, a course of events indicative of the activation of the intrinsic pathway of apoptosis and in agreement with another recent study of the SFV infection of primary mouse embryo fibroblasts (86). Importantly, in the absence of virus envelope proteins, ER stress is not activated, caspase activation is delayed, and cell death is slowed down (compared to whole-virus infection).

Despite the synchronous infection of virtually all cells in the culture, JC-1 staining indicated that not all cells lost their membrane potential synchronously. The viability of infected cultures declined progressively from 10 to 26 h postinfection. It is likely that cells responded at different rates to infection, which is consistent with the suggestion that the cell cycle affects alphavirus-induced apoptosis (38).

Mitochondrial membrane disruption very early in infection could result from a number of factors. One possibility is that SFV directly interacts with mitochondria as part of the virus life cycle. Some viruses, for example, flock house virus, replicate on the outer membranes of mitochondria, and rubella virus, a close relative of the alphaviruses, associates with mitochondria during replication (45, 56). However, there have been numerous electron microscopic studies of alphavirus replication with no reports associating virus replication with mitochondria (43). SFV replicates on endosome-like vacuoles (23). It is more likely that the disruption of mitochondrial membranes occurs as a result of the activation of proapoptotic events. Bak is known to cause cytochrome *c* release from mitochondria and disrupt MMP and recently has been shown to play an important role in SFV-induced apoptosis in primary mouse fibroblasts, and in BHK cells SINV apoptosis is induced by the related protein Bad (57, 86). It is not clear how alphaviruses activate these BH3 domain Bcl2 family proapoptotic proteins. One possibility is through the activation of p53 (90). However, SFV is not known to perturb nuclear events likely to activate p53, and p53 is not essential for SFV-induced cell death (29). Other more likely possibilities include the detection of virus RNA by RIG-I-like helicases, IPS-1-induced cell death, or the virus-induced disruption of cellular transcription or translation (13, 25, 34, 46, 64).

Apoptosis is activated by the replication of SFV replicons that do not contain the virus structural coding sequences, indicating that the virus structural proteins are not required for cell death (29). However, as we show here, their presence accelerates cell death. SFV is an enveloped virus, and the viral envelope glycoproteins are made on and traffic through the ER. Here, we demonstrate that the UPR of the ER is activated by infection with SFV, but not by infection with SFV VRPs lacking the virus structural protein-coding region. A number of other viruses also have been shown to activate the UPR, including West Nile, Tula, and vesicular stomatitis viruses (24, 49, 55). The West Nile virus (WNV) infection of neuronal cells results in XBP1 splicing, the phosphorylation of eIF-2α, and the upregulation of CHOP (55). Hepatitis C virus envelope glycoproteins expressed alone are sufficient to upregulate BIP expression and activate the UPR (9). Mutations in the SFV E2 glycoprotein reduce cytotoxicity, while the isolated expression of SINV glycoproteins triggers apoptosis (27, 39, 84). These effects may be mediated by the ER UPR.

The capsid protein of WNV can suppress the expression of OASIS, an ER stress-related protein (42, 88). This raises the possibility that alphavirus capsid proteins also affect ER stress. The capsid protein of new world alphaviruses such as Western and Venezuelan equine encephalitis viruses is known to play a significant role in the shutdown of transcription and translation in infected cells (4, 26). Using VRPs expressing nsPs and either capsid protein only or envelope proteins only, we specifically show that the envelope proteins are responsible for the activation of ER stress and that the capsid protein is not involved.

CHOP was upregulated by both SFV4 and VRP infection. CHOP has proapoptotic activity; it suppresses Bcl-2 expression, allowing proapoptotic proteins to trigger apoptosis (54). CHOP expression began between 7 and 9 h following infection with either SFV or VRPs. Therefore, while CHOP may contribute to apoptosis, it seems unlikely to be responsible for the

different rate of apoptosis between SFV4- and VRP-infected cells. CHOP upregulation in VRP-infected cells probably results from the activation of PKR. PKR activated by (viral) double-stranded RNA (dsRNA) phosphorylates eIF-2α, leading to ATF4 translation and CHOP upregulation (32). PKR is activated by SFV infection but is not required for SFV-induced apoptosis (6); indeed, PKR delays SFV-induced apoptosis, probably as a result of its inhibition of early events in virus replication (6). The activation of the UPR results in the activation of PERK, which also phosphorylates eIF-2α, resulting in the translation of ATF4 and CHOP (79). CHOP and ATF4 expression thus are a consequence of eIF-2α phosphorylation resulting from the activation of either PKR or PERK and therefore are not exclusive markers of ER stress. XBP1 splicing and caspase-12 activation are more specific markers of ER stress and were observed only in SFV4-infected, not VRP-infected, cells.

The primary route by which ER stress induces apoptosis is likely to be the release of Ca^{2+} from the ER. This builds up in the mitochondria, leading to membrane permeabilization and apoptosis (14). The activation of caspase-12, located on the cytoplasmic surface of the ER and activated by calpains as a result of calcium ion release (60, 66), is an agreed marker of this stress response, but the role of caspase-12 activation in apoptosis is controversial. Caspase-12 activation has been suggested to be essential for ER stress-induced apoptosis (61). However, more recent studies have shown that caspase-12 is more likely to play a role in the control of inflammation (73); caspase-12 negatively regulates caspase-1 (70) and NOD signaling (44). Using siRNA, we were unable to ablate caspase-12 expression, but a strong reduction in protein levels was attained. Following SFV infection, these cells died at the same rate as control cells, suggesting that activated caspase-12 is not required and does not contribute to cell death in response to ER stress initiated by SFV infection.

In summary, in NIH 3T3 cells, SFV infection triggers the intrinsic pathway of cell death, strongly upregulates CHOP, and activates the unfolded protein stress response of the ER. This ER stress response is activated by the virus envelope glycoproteins and not by virus capsid protein, and independently of caspase-12, it accelerates apoptotic cell death triggered by the replicon. Even for a relatively simple RNA virus, it is clear that cell death is triggered by more than one aspect of the virus-cell interaction; in the case of SFV, events independent of and dependent on virus envelope proteins are crucial.

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