NSm Protein of Rift Valley Fever Virus Suppresses Virus-Induced Apoptosis

Sungyong Won,1 Tetsuro Ikegami,1 C. J. Peters,1,2 and Shinji Makino1*

Departments of Microbiology and Immunology1 and Pathology,2 University of Texas Medical Branch at Galveston, Galveston, Texas 77555-1019

Received 6 June 2007/Accepted 22 September 2007

Rift Valley fever virus (RVFV) is a member of the genus Phlebovirus within the family Bunyaviridae. It can cause severe epidemics among ruminants and fever, myalgia, a hemorrhagic syndrome, and/or encephalitis in humans. RVFV has a single-stranded, tripartite RNA genome composed of the L, M, and S segments. The L segment is of negative polarity and encodes the RNA-dependent RNA polymerase (L). The S segment uses an ambisense strategy for gene expression; a nonstructural protein, NSs, is translated from the first AUG codon of the ORF, while the nucleocapsid (N) protein is translated from the pre-Gn region, i.e., NSm and the 78-kDa protein. The M gene open reading frame (ORF) in M mRNA contains five in-frame translation initiation codons within the preglycoprotein (pre-Gn) region, which is located upstream of the Gn and Gc genes (20, 26, 48). The 78-kDa protein is translated from the first AUG codon of the ORF, and its coding sequence includes the entire NSm and Gn coding sequences. NSm is translated from the region from the second AUG codon to the end of the pre-Gn region (7), strongly suggesting that there is selection pressure(s) to retain an RNA element(s) in the pre-Gn region and/or proteins encoded (fully or partially) by the pre-Gn region, i.e., NSm and the 78-kDa protein. Currently, the biological functions of the NSm and 78-kDa proteins of RVFV and proteins encoded by the pre-Gn regions of other phleboviruses are totally unclear, though a mutant RVFV lacking both NSm and 78-kDa protein expression showed attenuated virulence in rats (6), implying a possible role of the RVFV NSm and/or 78-kDa protein in viral pathogenesis.

In the present study, we have generated a deletion mutant of an attenuated MP-12 strain of RVFV, which expresses neither the NSm protein nor the 78-kDa protein, due to a large deletion in the pre-Gn region of the M segment. We found that cells infected with the deletion mutant underwent apoptotic cell death earlier than cells infected with the parental virus and that NSm expression inhibited the rapid apoptotic cell death in mutant virus-infected cells. NSm expression also suppressed staurosporine (STP)-induced apoptosis, demonstrating that NSm performed its antiapoptotic function in the absence of other viral proteins. This is the first demonstration of the biological function of the NSm protein of any phlebovirus.
MATERIALS AND METHODS

Cells and viruses. Vero E6 cells, human embryonic kidney 293 cells, and cells of the murine macrophage-like line J774.1 were maintained in Dulbecco's modified essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). BHK-T7-9 cells, which express T7 RNA polymerase (25), were grown in MEM-alpha containing 10% FBS, arMP-12, a recombinant MP-12 vaccine candidate strain of RVFV, and arMP-12-del21/384, carrying a deletion in the pre-Gn region of arMP-12, were generated using an RVFV reverse-genetics system (22, 51). Both viruses were grown, and their titers were determined by a plaque assay, in Vero E6 cells.

Plasmids and rescue of mutant virus. The entire NSm, 78-kDa, and 73-kDa ORFs (nucleotides [nt] 135 to 479, 21 to 290, and 174 to 2090, respectively) of the anti-viral-sense M segment were independently cloned into a pCAGGS plasmid (28), resulting in pCAGGS-NSm, pCAGGS-78, and pCAGGS-73, respectively. For the generation of pCAGGS-78, the second and third AUG codons of the NSm ORF were replaced with GCC to eliminate expression of the NSm and 73-kDa proteins. For the generation of arMP-12-del21/384, two EcoRI sites were created at nt 21 and 384 of the M gene ORF of a plasmid expressing an anti-viral-sense M segment. EcoRI digestion of this plasmid and subsequent self-ligation resulted in pPro-T7-avS(-del21/384). arMP-12 was recovered from BHK/T7-9 cells that were cotransfected with pPro-T7-avS(+), pPro-T7-avS(+), and pPro-T7-avL(+), all of which express anti-viral-sense RNA segments, pT7-IRESV-N, expressing N protein, pT7-IRESV-L, expressing L protein, and pCAGGS-Gp5 expressing proteins encoded in the M gene ORF, as described previously (22, 51). The rescued virus was amplified once in Vero E6 cells and used in the experiments.

Plaque assay. After adsorption of virus to Vero E6 cells at 37°C for 1 h, the inoculum was removed, and the cells were washed three times with phosphate-buffered saline and overlaid with modified Eagle's medium containing 0.6% tragacanth gum (MP Biomedicals, Inc.), 2.5% FBS, and 5% tryptose phosphate broth. At 4 days postinfection (p.i.), the overlay was removed, and cells were fixed and stained with 0.75% crystal violet, 10% formaldehyde, and 5% ethanol.

Northern blot analysis. Intracellular RNAs were extracted from virus-infected cells using TRIzol reagent (Invitrogen). Approximately 100 ng of RNA was denatured and separated on 1% denaturing agarose-formaldehyde gels and transferred to a nylon membrane (Roche Applied Science). Northern blot analysis was performed with strand-specific RNA probes as described previously (23).

Antibodies. An anti-NSm rabbit polyclonal antibody and an anti-RVFV mouse polyclonal antibody were used to detect viral proteins (51). A rabbit polyclonal antibody against an L protein peptide was prepared by immunizing rabbits with the synthetic peptide N-RDRSKQPFSPDHD, corresponding to amino acids 434 to 446 of the L protein.

Western blot analysis. Virus-infected and mock-infected cells were lysed with sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride (Bio-Rad) membrane. After being blocked for 1 h, the membranes were incubated with an anti-NSm antibody or preimmune serum. Radioimmunoprecipitation. Virus-infected and mock-infected cells were radiolabeled with 100 μCi/ml of Tran35S-label (MP Biomedicals) at 8 h p.i. for 30 min. Cytoplasmic extracts were prepared as described previously (51) and incubated with an anti-NSm antibody or preimmune serum. Radioimmunoprecipitation was performed as described previously (51).

Cell viability assay. Confluent Vero E6 cells grown in 24-well plates were either mock infected or independently infected with arMP-12 or arMP-12-del21/384 at a multiplicity of infection (MOI) of 10. Cell viability was determined by using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)-based in vitro toxicity assay kit (Sigma), which measures the activity of mitochondrial dehydrogenases in living cells. At various times postinfection, MTT (0.5 mg/ml) was added to each well for 4 h, and the reaction was terminated by adding an MTT solubilization solution. After MTT formazan crystal was completely dissolved, the absorbance of samples was measured at a wavelength of 570 nm and the background absorbance at 690 nm was subtracted. The viability of the infected cells was determined as a percentage of the viability of mock-infected cells at the corresponding time p.i.

Annexin V/PI staining and flow cytometry. Vero E6 cells were either mock infected or independently infected with arMP-12 or arMP-12-del21/384 at an MOI of 3. All cells in the cultures, including floating cells, were collected at 20 and 40 h.p.i. The harvested cells were washed three times and then stained with annexin V-fluorescein isothiocyanate and propidium iodide (PI) for 15 min at room temperature using the Annexin V-FITC apoptosis detection kit I (BD Pharminogen). We then analyzed 1 × 10^6 stained cells by flow cytometry (BD FACSCanto flow cytometer; Becton Dickinson).

Caspase enzymatic activity assays. The caspase enzymatic activities of virus-infected cells were evaluated via caspase colorimetric assay kits (BioVision, Mountain View, CA). In this assay, approximately 1 × 10^6 cells were washed with phosphate-buffered saline and lysed with 50 μl of chilled cell lysis buffer for 10 min on ice. After centrifugation for 1 min at 10,000 × g, supernatants (cytosolic extracts) were collected and protein concentration measured by the Bradford assay. Fifty or 100 μg of proteins was diluted in 50 μl of cell lysis buffer and 50 μl of 2× reaction buffer for each assay. The activity of each caspase was determined with colorimetric peptide substrates: DEVD-pNA (chromophore p-nitroanilide) for caspases 3 and 7 (caspase-3/7), IETD-pNA for caspase-8, and LEHD-pNA for caspase-9. Each sample was incubated with the peptide substrate for 2 h, and color changes were measured at 405 nm in a spectrophotometer (Bio-Rad) using a quartz microcuvette. The background reading from cell lysates and buffers was subtracted from the sample reading.

Reagents. An anti-cleaved caspase-3 (Asp175) antibody and an anti-poly-(ADP-ribose) polymerase (PARP) antibody were purchased from Cell Signaling Technology (Danvers, MA). STP and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO), and Z-IRETD-FMK (a caspase-8 inhibitor) and Z-LEHD-FMK (a caspase-9 inhibitor) were purchased from BD Biosciences (San Jose, CA).

Statistical analysis. A paired Student t test was used to compare significance. A P value of <0.05 was considered statistically significant.

RESULTS

Generation of arMP-12-del21/384 carrying a large deletion in the pre-Gn region of the M segment. The pre-Gn region of...
the RVFV M gene ORF contains five in-frame start codons, and the 78-kDa and NSm proteins are translated from the first and second AUG codons, respectively (Fig. 1A) (26, 48, 51). We previously reported that neither the 78-kDa protein nor the NSm protein is detected in cells infected with arMP-12-delNSm/78, which carries substitution mutations in the first and second AUG codons. This mutant virus and the parental virus, arMP-12, form plaques similar in size and morphology and have comparable growth kinetics in several cell lines (51). However, we unexpectedly detected an additional protein(s) with a molecular mass of 73 to 75 kDa, which is recognized by the anti-NSm antibody, in arMP-12-delNSm/78- and arMP-12-infected cells (51). To completely eliminate the accumulation of detectable viral proteins whose coding sequences contain the pre-Gn region in virus-infected cells, we generated another mutant virus, arMP-12-del21/384, with a deletion from nt 21 to 384 in the pre-Gn region, by using a reverse-genetics system (22, 51) (Fig. 1A). Sequence analysis showed that the rescued arMP-12-del21/384 virus retained the introduced deletion in the pre-Gn region and had no other mutations in the M segment. In contrast to arMP-12-delNSm/78, which produces plaques indistinguishable from those made by arMP-12, arMP-12-del21/384 produced plaques that were larger than those produced by arMP-12 in Vero E6 cells (Fig. 1B). We have successfully recovered arMP-12-del21/384 in three independent rescue experiments, and all rescued viruses produced plaques that were similar in size and morphology; however, they were larger than those produced by arMP-12 in Vero E6 cells (Fig. 1B). We have successfully recovered arMP-12-del21/384 in three independent rescue experiments, and all rescued viruses produced plaques that were similar in size and morphology; however, they were larger than those produced by arMP-12 in Vero E6 cells (Fig. 1B). We have successfully recovered arMP-12-del21/384 in three independent rescue experiments, and all rescued viruses produced plaques that were similar in size and morphology; however, they were larger than those produced by arMP-12 in Vero E6 cells (Fig. 1B).
Analysis of the accumulation of viral RNAs and proteins and the growth kinetics of arMP-12-del21/384. Northern blot analysis revealed the accumulation of similar levels of viral sense RNAs in arMP-12-infected and arMP-12-del21/384-infected Vero E6 cells (Fig. 2A). The two viruses also accumulated similar amounts of anti-viral-sense RNAs (data not shown). Western blot analyses showed no substantial differences in the amounts of L, Gn/Gc, NSs, and N proteins between arMP-12-infected and arMP-12-del21/384-infected Vero E6 cells (Fig. 2B); a modest increase in the accumulation of Gn/Gc, N, and NSs proteins in arMP-12-del21/384-infected cells relative to that in arMP-12-infected cells, shown in Fig. 2B, was not reproducible. Radioimmunoprecipitation analysis of infected-cell lysates with an anti-NSm antibody showed accumulations of the 78-kDa protein, NSm protein, and a viral protein with a molecular mass of 75 kDa (the 75-kDa protein) in arMP-12-infected cells, whereas these proteins did not accumulate in arMP-12-del21/384-infected cells (Fig. 2B and C). A similar radioimmunoprecipitation analysis of arMP-12-delNSm/78-infected cells demonstrated selective immunoprecipitation of a viral protein with a molecular mass of 73 kDa (the 73-kDa protein). arMP-12 and arMP-12-del21/384 showed similar growth kinetics in Vero E6 cells after infection at an MOI of 1 (Fig. 2D). They also replicated with similar kinetics in 293 cells, MRC-5 cells, and cells of the murine macrophage-like line J774.1 (4) (after infection at an MOI of 1 or 0.01 (Fig. 2D and data not shown). These data are consistent with previous findings that the lack of NSm and 78-kDa protein accumulation and the presence of a large deletion at the pre-Gn region do not affect virus replication efficiency in cell cultures (18, 51).

arMP-12-del21/384 replication induces more-extensive apoptotic cell death than arMP-12 replication. arMP-12-del21/384 and arMP-12 replicated with similar kinetics in cell cultures, yet the former produced larger plaques than the latter (Fig. 1B). These data led us to hypothesize that arMP-12-del21/384 replication may induce more-extensive cell death than arMP-12 replication. To test this hypothesis, Vero E6 cells were independently infected with arMP-12, arMP-12-delNSm/78, or arMP-12-del21/384 at an MOI of 10, and cell viabilities were determined by an MTT-based cytotoxicity assay, which measures the mitochondrial dehydrogenase activities of live cells. Cell viabilities of infected cell cultures were expressed as percentages of the absorbance of a mock-infected cell culture at the corresponding time p.i. (Fig. 3A). All infected cell cultures showed similar levels of cell viability during the first 24 h p.i., whereas the viabilities of arMP-12-del21/384-infected cells were significantly lower than those of arMP-12-infected cells from 48 h p.i. to 72 h p.i.; at 60 h p.i., the cell viabilities of arMP-12-del21/384-infected cultures were about 40% less than those of arMP-12-infected cell cultures. The kinetics of cell viability for arMP-12-delNSm/78-infected and arMP-12-infected cells were similar throughout infection, except at 24 h p.i., when marginally lower cell viabilities were observed for the former.

To determine whether arMP-12-del21/384 replication induced more-extensive apoptotic cell death than arMP-12 replication, annexin V/PI staining and a subsequent flow cytometry analysis were performed. Annexin V is a Ca\(^{2+}\)-dependent phospholipid-binding protein with a high affinity for phosphatidylinerine, which is translocated from the inner to the outer leaflet of the plasma membrane in the early stage of apoptosis (38). PI, which intercalates into double-stranded DNAs, is excluded by viable cells but can penetrate the membranes of dying or dead cells (50). Accordingly, annexin V-positive and PI-negative cells are those undergoing early apoptosis. Vero E6 cells were either mock infected or independently infected with arMP-12 or arMP-12-del21/384 at an MOI of 3. An immunofluorescence assay that detected viral proteins showed that nearly 100% of Vero E6 cells and 293 cells were infected with viruses at an MOI of 3 (data not shown). At 20 and 40 h p.i., all cells, including floating cells, were collected from the cultures and stained with annexin V/PI; then they were analyzed by flow cytometry (Fig. 3B). The lower-right quadrant (Q4) of Fig. 3B represents early-apoptotic cells, i.e., annexin V-positive, PI-negative cells. At 20 h p.i., mock-infected cells and arMP-12-infected cells had 5.9% and 8.4% apoptotic cells, respectively, whereas 20.7% of arMP-12-del21/384-infected cells underwent apoptosis, demonstrating that the number of cells at the early stage of apoptosis was substantially higher for arMP-12-del21/384-infected cells than for arMP-12-infected cells. By 40 h p.i., the apoptotic cell populations had increased substantially in infected cells, and the apoptotic cell population in arMP-12-del21/384-infected cells (35.6%) was higher than that in arMP-12-infected cells (22.3%). Furthermore, the population of cells at the late stages of apoptosis and/or necrosis, shown in the upper-right quadrant (Q2), had increased in infected cells by 40 h p.i., and this population was larger for arMP-12-del21/384-infected cells (24.2%) than for arMP-12-infected cells (14.8%). These data suggested that the NSm and/or 78-kDa protein suppressed virus-induced apoptotic cell death.

Status of caspase activation in arMP-12- and arMP-12-del21/384-infected cells. It is well established that once apoptosis is triggered, upstream caspases, e.g., caspase-8 or -9, induce the cleavage of caspase-3, yielding active cleaved forms of caspase-3. FIG. 3. Cell viability assay (A) and annexin V/PI staining and flow cytometric analysis (B) of infected cells. (A) Vero E6 cells were independently infected with arMP-12, arMP-12-delNSm/78, or arMP-12-del21/384 at an MOI of 10. At the indicated time p.i., infected cells were treated with 0.5 mg of MTT/ml and incubated for 4 h. The metabolic reaction was terminated, and MTT formazan crystals in living cells were dissolved by addition of an MTT solubilization solution. Absorbance was measured at a wavelength of 570 nm, and the background absorbance at 690 nm was subtracted. Cell viability was calculated as a percentage relative to the absorbance of mock-infected control cultures at the corresponding time p.i. Data are the results of triplicate experiments. (B) Vero E6 cells were either mock infected or independently infected with arMP-12 or arMP-12-del21/384 at an MOI of 3. Cells, including floating cells, were collected at 20 and 40 h p.i. and were stained with annexin V and PI for 15 min. A total of 1 × 10⁶ stained cells were analyzed by flow cytometry. Dot plots are divided into quadrants of cell populations: Q1 (staining PI positive and annexin V negative), Q2 (staining positive with both), Q3 (staining negative with both), and Q4 (staining annexin V positive and PI negative). Q4 represents the early-apoptotic cell population. The percentage of cells in each quadrant is also presented.
caspase-3 (p17/p19), which in turn cleave the downstream cellular substrates, e.g., PARP, to execute apoptosis (33). To further establish that arMP-12-del21/384 replication induced a level of apoptotic cell death more extensive than that induced during arMP-12 replication, we examined the activation status of caspase-3 in arMP-12- and arMP-12-del21/384-infected cells. Vero E6, 293, and J774.1 cells were either mock infected or infected with arMP-12 or arMP-12-del21/384 at an MOI of 3. Total-cell lysates were collected at the indicated time p.i. and subjected to Western blot analysis. Anti-cleaved caspase-3 (Asp175), an anti-PARP antibody, and an anti-actin antibody were used to demonstrate cleaved caspase-3 (p17), both uncleaved and cleaved PARP, and actin, respectively. RVFV N and NSs proteins were detected by an anti-RVFV antibody. Lanes STP and DMSO, 293 cells treated with 3 μM STP in DMSO and with DMSO only, respectively. The specific band signal in each immunoblot was quantified by densitometric scanning and normalized against an actin control for cleaved caspase-3 or against total PARP (uncleaved and cleaved PARP) for cleaved PARP. Increases (n-fold) over the signal from mock-infected cells are given below the lanes.

FIG. 4. Activation of caspase-3 and cleavage of PARP in virus-infected cells. Vero E6, 293, and J774.1 cells were either mock infected or infected with arMP-12 or arMP-12-del21/384 at an MOI of 3. Total-cell lysates were collected at the indicated time p.i. and subjected to Western blot analysis. Anti-cleaved caspase-3 (Asp175), an anti-PARP antibody, and an anti-actin antibody were used to demonstrate cleaved caspase-3 (p17), both uncleaved and cleaved PARP, and actin, respectively. RVFV N and NSs proteins were detected by an anti-RVFV antibody. Lanes STP and DMSO, 293 cells treated with 3 μM STP in DMSO and with DMSO only, respectively. The specific band signal in each immunoblot was quantified by densitometric scanning and normalized against an actin control for cleaved caspase-3 or against total PARP (uncleaved and cleaved PARP) for cleaved PARP. Increases (n-fold) over the signal from mock-infected cells are given below the lanes.
control for apoptosis induction. As shown in Fig. 4, STP treatment induced accumulation of cleaved caspase-3 and PARP, while neither was accumulated in cells treated with DMSO alone. In both 293 and Vero E6 cells, throughout infection, cleaved caspase-3 was more abundant in arMP-12-del21/384-infected cells than in arMP-12-infected cells. For 293 cells, caspase-3 cleavage was detected as early as 8 h p.i. in arMP-12-del21/384-infected cells, whereas only a low level of cleaved caspase-3 was detectable at 16 h p.i. in arMP-12-infected cells. Likewise, the levels of PARP cleavage were higher in mutant virus-infected cells than in cells infected with the parental virus, arMP-12. We noted efficient accumulation of cleaved caspase-3 and PARP at 6 h p.i. in arMP-12-del21/384-infected J774.1 cells but not in arMP-12-infected J774.1 cells, whereas these differences were no longer obvious at 8 h p.i. Western blotting using an anti-RVFV antibody showed that accumulation of N and NSs proteins was similar for arMP-12-infected and arMP-12-del21/384-infected cells, a finding that, together with the identical growth curves (Fig. 2D), suggested that increased caspase-3 activation in arMP-12-del21/384-infected cells was not due to the efficient replication of this virus.

To further confirm that the cleaved caspase-3 in arMP-12-del21/384-infected cells was bioactively, caspase-3/7 activities in arMP-12- and arMP-12-del21/384-infected cells were measured by using their colorimetric substrate, DEVD-pNA. The levels of caspase-3/7 activities in mutant virus-infected cells were substantially higher than those in parental-virus-infected cells at 16 h p.i. for both Vero E6 and 293 cells (Fig. 5A) (P < 0.001). In contrast, only an insignificant difference in caspase-3/7 activities was detected between mock-infected cells and arMP-12-infected cells.

Next, we determined the enzymatic activities of the upstream caspsases, i.e., caspase-8 (Fig. 5B) and caspase-9 (Fig. 5C), at 16 h p.i. in infected Vero E6 and 293 cells. In both cell lines, caspase-8 activities were higher in arMP-12-del21/384-infected cells than in arMP-12-infected cells; these differences were statistically significant (P < 0.001). Caspase-8 activities in arMP-12-infected cells were also statistically significantly higher than those in mock-infected cells (P < 0.001 for Vero E6 cells and P < 0.005 for 293 cells). Caspase-9 activities were statistically significantly higher in arMP-12-del21/384-infected cells than in arMP-12-infected cells (P < 0.05 for both cell lines), whereas the differences between mock-infected and arMP-12-infected cells, and those between mock-infected and arMP-12-del21/384-infected cells, were not statistically significant in either cell line. These data suggested that activated caspase-8 primarily induced caspase-3 cleavage in arMP-12-del21/384-infected cells. Overall, our data were consistent with the notion that the viral proteins encoded by the pre-Gn region, including the NSm and/or 78-kDa protein, suppress the activities of caspase-8 and its downstream caspase, caspase-3, causing a delay in apoptotic cell death in arMP-12-infected cells.

Expression of the NSm and 73-kDa proteins suppresses caspase-3 cleavage and activation in arMP-12-del21/384-infected cells. To investigate whether the viral proteins whose coding sequences contain the pre-Gn region, i.e., NSm, the 73-kDa protein, and the 78-kDa protein, could inhibit apoptotic cell death in RVFV-infected cells, 293 cells were independently transfected with a eukaryotic expression plasmid carrying the NSm protein ORF (pCAGGS-NSm), the 78-kDa protein ORF (pCAGGS-78), or the 73-kDa protein ORF (pCAGGS-73), or with a control plasmid, pCAGGS. In our hands, the transfection efficiencies achieved in 293 cells were about 70 to 80% (data not shown). At 48 h posttransfection, cells were either mock infected or independently infected with arMP-12 or arMP-12-del21/384 at an MOI of 3. Total-cell lysates were collected at 16 h p.i. and subjected to Western blot analysis in order to compare the levels of caspase-3 cleavage (Fig. 6A). It was evident that transfection of pCAGGS-NSm and pCAGGS-78 resulted in efficient accumulation of NSm protein and the 78-kDa protein, respectively; the abundance of NSm protein in transfected cells was higher than that in arMP-
12-infected cells. Transfection of pCAGGS-73 resulted in a modest amount of 73-kDa protein accumulation. In arMP-12-infected cells (Fig. 6, lanes 5 to 8), the expression of all three proteins had little effect on the abundance of cleaved caspase-3 (p17), whereas expression of the NSm and 73-kDa proteins clearly suppressed p17 accumulation in arMP-12-del21/384-infected cells (Fig. 6, lanes 10 and 12). In contrast, expression of the 78-kDa protein had little effect on p17 accumulation in arMP-12-del21/384-infected cells (Fig. 6, lane 11). Consistent with those data, caspase-3/7 activities in arMP-12-del21/384-infected cells that expressed NSm protein or the 73-kDa protein, but not the 78-kDa protein, were statistically significantly lower than those in arMP-12-del21/384-infected cells expressing NSm protein, pCAGGS-73 (p17), or pCAGGS-78 at an MOI of 3. Total-cell lysates were collected at 16 h p.i. and analyzed by Western blot analysis with an anti-cleaved caspase-3 antibody detecting cleaved caspase 3 (p17), an anti-NSm antibody detecting NSm and the 73-kDa and 78-kDa proteins, and an anti-actin antibody. Experiments were performed three times, and similar results were obtained. A set of representative data is shown. (B) 293 cells in six-well plates were transfected with 2.5 μg of plasmids as described for panel A and then either mock infected or independently infected with arMP-12 or arMP-12-del21/384 at 48 h posttransfection. At 16 h p.i., cell lysates were collected and the caspase-3/7 enzymatic activities of lysates were analyzed as described in the legend to Fig. 5. All samples were prepared in triplicate, and statistical significance was determined by a paired Student t test (*, P < 0.001; #, P < 0.05).

These experiments demonstrated that the independently expressed NSm and 73-kDa proteins, but not the 78-kDa protein, from the transfected plasmids worked in trans to suppress caspase-3 activation in arMP-12-del21/384-infected cells; the NSm and 73-kDa proteins indeed inhibited apoptotic cell death in RVFV-infected cells.

NSm protein expression suppresses STP-induced apoptosis. To further establish an antiapoptotic function of the NSm protein, we examined whether NSm protein expression suppressed STP-induced apoptosis. STP activates both caspase-8 and caspase-9 (45), while the exact mechanism by which STP induces apoptosis is unclear. 293 cells were independently transfected with pCAGGS or pCAGGS-NSm, followed by treatment with 3 μM STP at 48 h posttransfection. Cell extracts were prepared at 3 h after STP treatment, and the
enzyomatic activities of caspase-8 and caspase-9 were measured (Fig. 7). Consistent with our expectation, STP treatment induced activation of both caspases. It was evident that NSm protein expression efficiently suppressed STP-induced activation of both caspase-8 and caspase-9. NSm protein was quite competent to suppress STP-induced caspase-8 and -9 activities, as evidenced by the fact that its inhibitory effects were comparable to those of the synthetic inhibitors of caspase-8 (Z-IETD) or caspase-9 inhibitor (Z-LEHD) 1 h prior to STP treatment. Asterisks represent P values of <0.001 for panel A and <0.01 for panel B. Data are results of triplicate experiments.

DISCUSSION

The RVFV NSs protein, 78-kDa protein, and NSm protein are viral accessory proteins, because they are dispensable for virus replication in cell cultures (22, 51). A biological function of NSs, which suppresses host mRNA transcription, has been described (5, 34), whereas the biological functions of the NSm and 78-kDa proteins were unclear. By using arMP-12-del12/384, carrying a deletion in the pre-Gn region of the M gene ORF, and its parental virus, the present study identified RVFV NSm protein as an inhibitor of apoptosis.

Among five in-frame AUG codons in the pre-Gn region of RVFV M mRNA, the fourth AUG codon is used mainly for translation of the Gn and Gc proteins, while the 78-kDa and NSm proteins are translated from the first and second AUG codons, respectively (27, 47). In addition to these proteins, the present study and our previous studies (51) showed an accumulation of the 75-kDa protein, which reacted with an anti-NSm antibody, in MP-12-infected cells. Synthesis of a viral protein from the second AUG codon that corresponds to the 75-kDa protein was also reported for cells expressing RVFV M segment RNA (20). We also detected an accumulation of the 73-kDa protein, which also reacted with the anti-NSm antibody, in cells supporting the replication of arMP-12-delNSm/78; the NSm, 78-kDa, and 75-kDa proteins were not synthesized in arMP-12-delNSm/78-infected cells (Fig. 2C). Our unpublished studies showed that replication of the MP-12 mutant carrying a substitution mutation at the third AUG codon resulted in accumulation of the NSm, 78-kDa, and 75-kDa proteins but not of the 73-kDa protein. These data suggested that the 73-kDa and 75-kDa proteins were translated from the third and second AUG codons, respectively, and that the 73-kDa protein accumulation occurred when the first and/or second AUG codon was abolished. Among those proteins whose coding sequences contain the pre-Gn region, the NSm and 73-kDa proteins, but not the 78-kDa protein, suppressed caspase-3 cleavage and activation in the trans-complementation experiments (Fig. 6). Consistent with these data, the kinetics of cell viability were similar for arMP-12-infected cells and cells infected with arMP-12-delNSm/78, which expressed the 73-kDa protein but not the 78-kDa, 75-kDa, and NSm proteins (Fig. 3A). These data revealed that the NSm and 73-kDa proteins, but not the 78-kDa protein, had an antiapoptosis function. Furthermore, our data suggested that a low abundance of the 73-kDa protein could exert the antiapoptotic activity (see Fig. 2 and 6). Then why did the 78-kDa protein, which includes the sequences that are present in NSm and the 73-kDa protein, not perform an antiapoptotic function? It is possible to speculate that the 78-kDa protein translocates into the lumen of the endoplasmic reticulum through its N-terminal signal peptide, whereas the NSm, 75-kDa, and 73-kDa proteins lack an N-terminal signal peptide, and hence, the portions of these proteins encoded by the pre-Gn region are probably localized within the cytoplasm (19, 20, 26). If the NSm and 73-kDa proteins exert their antiapoptotic activities in the cytoplasm, then it is not surprising that the 78-kDa protein could not perform an antiapoptotic function, since its pre-Gn region may be located within the lumen.

We reported that arMP-12 and its three different mutant viruses, one lacking NSm protein expression, the second lacking 78-kDa protein expression, and the third lacking the expression of both the NSm and 78-kDa proteins, produce plaques with the same sizes and morphologies (51). Because the 73-kDa and/or 75-kDa protein accumulates in cells infected with these mutant viruses (51), we suspect that expression of the 73-kDa and/or 75-kDa protein in the cells infected with these mutant viruses exerted antiapoptotic activities and contributed to the formation of plaques indistinguishable from those produced by arMP-12. Gerrard et al. reported the generation of the ZH501 strain of RVFV lacking most of the pre-Gn region (18). Like arMP-12-del12/384, this mutant virus
most probably does not express any viral proteins whose coding sequences carry the pre-Gn region, yet Gerrard et al. reported that this mutant produced plaques of the same size and morphology as those of its parental wild-type RVFV. It should be noted that 0.6% tragacanth gum was used as an overlay in our plaque assay, while Gerrard et al. used 1% agarose for the overlay. It is possible that the tragacanth gum overlay was more sensitive at detecting minute differences in RVFV plaque sizes and morphologies.

There are ample examples of apoptotic cell death occurring in virus infection; apoptosis is induced by host immune responses, virus-encoded proapoptotic proteins, or double-stranded RNA-mediated host cell responses (2, 24, 40). Induction of apoptosis in virus infection is considered to be one of the host defense mechanisms eliminating infected cells to limit viral replication and spread. Several bunyaviruses induce apoptosis in cell cultures and in vivo (9, 13, 14, 36). To secure efficient production of progeny virus, viruses have developed various strategies to suppress apoptosis by expressing viral antiapoptotic proteins (40, 43). The virus-mediated inhibition of apoptosis also contributes to the establishment of latent infections or viral oncogenesis. Large DNA viruses developing low levels of cytolysis, including adenoviruses, papovaviruses, herpesviruses, poxviruses, and baculoviruses (11, 17, 40, 43, 44, 49), have been shown to carry more than one antiapoptotic gene. In contrast to the work on large DNA viruses, studies of antiapoptotic proteins in RNA viruses are rather limited; several past studies have suggested the presence of antiapoptotic proteins in several picornaviruses (10), hepatitis C virus (41), respiratory syncytial virus (8), Bunyamwera virus (29), and influenza A virus (15). RVFV NSm protein is the first viral protein in phleboviruses to show an antiapoptosis function. Further studies are required to assess whether other bunyaviruses also encode antiapoptotic functions.

The number of dead cells among arMP-12-del21/384-infected cells was substantially higher than that among arMP-12-infected cells after 48 h p.i. (Fig. 3A), while the two viruses showed similar virus growth kinetics (Fig. 2D). Because efficient release of progeny RVFV occurs within the first 24 h after infection at an MOI of 1 or higher in cell cultures (Fig. 2D) (22, 51), the majority of progeny viruses have been released prior to virus-induced apoptosis. Rapid virus replication prior to induction of apoptotic cell death is one of the strategies that some RNA viruses have employed to secure efficient progeny production (30, 31), and it appears that RVFV also uses this strategy to circumvent the negative effects of virus-induced apoptosis for virus production. If RVFV always replicates quickly prior to the execution of apoptotic cell death, then why is the pre-Gn region in the RVFV M segment strictly retained in all RVFVs thus far sequenced (7)? A virus such as RVFV with such a rapid progression to cell death in virtually all viretate cell types infected might require prolongation of the time of productive infection. We also know that when cells that are undergoing transcriptional and/or translational suppression are exposed to tumor necrosis factor alpha, they quickly die by apoptosis (1, 46). It is established that RVFV replication efficiently suppresses host transcription and translation (22, 34). Accordingly, we suspect that a combination of virus replication and exposure of the infected cells to some extracellular factors, e.g., tumor necrosis factor alpha, may induce rapid apoptosis prior to the completion of maximum progeny virus production in RVFV-infected mammals; without NSm, RVFV may not be able to amplify efficiently in an infected host. Consistent with this notion, a recombinant wt RVFV lacking most of the pre-Gn region has attenuated virulence in rats (6), suggesting that proteins encoded by the pre-Gn region have some role in RVFV virulence.

Our data suggested that RVFV infection triggered apoptosis mainly through activation of caspase-8, which is a death receptor-mediated, extrinsic, apoptotic pathway, and that NSm expression suppressed caspase-8 activation (Fig. 5B). We also observed that NSm efficiently inhibited STP-induced caspase-8 and caspase-9 activities (Fig. 7). Because it is well established that activated caspase-8 can activate caspase-9 by provoking the release of cytochrome c from mitochondria via the cleavage of Bid (35, 37), it is conceivable that NSm protein inhibited either activated caspase-8 activity or an apoptotic pathway that activates caspase-8, resulting in the suppression of caspase-9 activation as well. However, we cannot exclude the possibility that the NSm protein also directly inhibits the activation of downstream caspases, including caspase-9 and caspase-3.

**ACKNOWLEDGMENTS**

This work was supported by grants to S.M. and C.J.P. from the Western Regional Center of Excellence for Biodefense and Emerging Infectious Diseases, NIH grant U54 AI057156, NIH-NIAID-DMID-02-24 Collaborative Grant on Emerging Viral Diseases, and DHS grant NOOO14-04-1-0660, U.S. Department of Homeland Security, National Center for Foreign Animal and Zoonotic Disease Defense. S.W. and T.I. were supported by the James W. McLaughlin Fellowship Fund.

**REFERENCES**


