Rotavirus Enterotoxin Nonstructural Protein 4 Targets Mitochondria for Activation of Apoptosis during Infection*\[5\]

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Background: Rotaviral nonstructural protein 4 (NSP4) disrupts Ca\(^{2+}\) ion homeostasis by translocating to the endoplasmic reticulum.

Results: In this study, we show translocation of NSP4 to mitochondria, dissipation of mitochondrial potential, and initiation of apoptosis, which NSP1 counteracts during early infection.

Conclusion: NSP4 and NSP1 regulate apoptosis during infection.

Significance: Study signifies modulation of cellular survival and apoptotic machinery by rotavirus for their own benefit.

Viruses have evolved to encode multifunctional proteins to control the intricate cellular signaling pathways by using very few viral proteins. Rotavirus is known to express six nonstructural and six structural proteins. Among them, NSP4 is the enterotoxin, known to disrupt cellular Ca\(^{2+}\) homeostasis by translocating to endoplasmic reticulum. In this study, we have observed translocation of NSP4 to mitochondria resulting in dissipation of mitochondrial membrane potential during virus infection and NSP4 overexpression. Furthermore, transfection of the N- and C-terminal truncated NSP4 mutants followed by analyzing NSP4 localization by immunofluorescence microscopy identified the 61–83 amino acid region as the shortest mitochondrial targeting signal. NSP4 exerts its proapoptotic effect by interacting with mitochondrial proteins adenine nucleotide translocator and voltage-dependent anion channel, resulting in dissipation of mitochondrial potential, release of cytochrome c from mitochondria, and caspase activation. During early infection, apoptosis activation by NSP4 was inhibited by the activation of cellular survival pathways (PI3K/AKT), because PI3K inhibitor results in early induction of apoptosis.

However, in the presence of both PI3K inhibitor and NSP4 siRNA, apoptosis was delayed suggesting that the early apoptotic signal is initiated by NSP4 expression. This proapoptotic function of NSP4 is balanced by another virus-encoded protein, NSP1, which is implicated in PI3K/AKT activation because overexpression of both NSP4 and NSP1 in cells resulted in reduced apoptosis compared with only NSP4-expressing cells. Overall, this study reports on the mechanism by which enterotoxin NSP4 exerts cytotoxicity and the mechanism by which virus counteracts it at the early stage for efficient infection.

Rotavirus, a nonenveloped double-stranded RNA virus belonging to the family Reoviridae, is the major cause of severe gastroenteritis in children under the age of 5 years and causes 2 million hospitalizations and 450,000 deaths per year worldwide (1). The virus with its 11 segmented dsRNA genome encodes six structural proteins (VP1 to VP4, VP6, and VP7), which form the virion, and six nonstructural proteins (NSP1 to NSP6), which are translated only in host cells after virus infection and do not form part of the mature infectious virus (2). Nonstructural proteins play a crucial role in infection by establishing host virus interaction by carrying out diverse function. Among them, NSP4 is identified as a virus enterotoxin (3). NSP4 is a 175-amino acid-long protein having a fundamental role in both viral morphogenesis and pathogenesis (3, 4). It was reported that exogenous addition of NSP4 can induce age- and dose-dependent diarrhea in a mouse model (5). NSP4 localizes in the endoplasmic reticulum and acts as an intracellular receptor of the double-layered virus particle during the entry of subviral particles into the endoplasmic reticulum (6–9). NSP4 has a membrane destabilizing activity due to its viroporin domain by which it can create a transmembrane aqueous pore in endoplasmic reticulum and increase the cytoplasmic Ca\(^{2+}\) concentration (10). An increase in cytoplasmic Ca\(^{2+}\) concentration and activation of Bax has been shown to play a role in rotavirus-induced cell death (11–13). It is natural for virus, an obligatory
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**EXPERIMENTAL PROCEDURES**

**Ethics Statement**—This investigation was approved by the Institutional Animal Ethics Committee, National Institute of Cholera and Enteric Diseases, Indian Council of Medical Research (registration number NICED/CPCSEA/AW/215/2012-IAEC/SSO and approval number 65/20/08/2009), registered under “Committee for the Purpose of Control and Supervision of Experiments on Laboratory Animals,” Ministry of Environment and Forests, Government of India, and it conforms with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health Publication 85-23, revised 1996.

**Viruses, Cells, and Virus Infection**—The monkey kidney cells (MA104), 293T cells, and HeLa cells were cultured in minimal essential medium, Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 2 mM sodium pyruvate, and 1× penicillin, streptomycin, and fungizone, respectively, in a 37 °C humidified incubator with 5% CO2. The simian rotavirus strain SA11 and NSP1 mutant strain A5-16 were used in this study. For infection, viruses were activated with acetylated trypsin (10 μg/ml) at 37 °C for 30 min and added to phosphate-buffered saline (PBS)-washed cells at an indicated multiplicity of infection (MOI).

**Construction of Vectors**—Vectors were constructed using the primers having specific restriction sites (Table 1).

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**Table 1**

| Vector          | Gene | RE site | Primer sequence                  |
|-----------------|------|---------|----------------------------------|
| pcDNA6/v5 (Invitrogen) | NSP4 | EcoRI   | 5' - GAAATTCGTTTCCAGCAGTCCACAC - 3' |
| pcDNA6/v5 (Invitrogen) | VDAC1 | XhoI    | 5' - CTGCTGATTGGATGCTTTGCTTAC - 3' |
| pcDNA6/v5 (Invitrogen) | ANT3 | EcoRI   | 5' - GAAATTCGTTTCCAGCAGTCCACAC - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 | Sall    | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 Δ1–25 | Sall | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 Δ1–48 | Sall | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 Δ1–60 | Sall | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 Δ1–83 | Sall | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 Δ143–175 | Sall | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 Δ92–175 | Sall | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 Δ74–175 | Sall | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 (61–91) | Sall | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 (61–83) | Sall | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 (74–91) | Sall | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 | EcoRI   | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |
| pAcGFP1-C2 (Clontech) | NSP4 | EcoRV   | 5' - GTCGACCACTTCTGCACTTCTTCCT - 3' |

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7 The abbreviations used are: VDAC, voltage-dependent anion channel; NSP, nonstructural protein; MTS, mitochondria translocating signal; m.o.i., multiplicity of infection; hpi, hours post infection; ER, endoplasmic reticulum; IVT, in vitro coupled transcription and translation; ANT, adenine nucleotide translocator; cyt c, cytochrome c; Z, benzoyloxy carbonyl; fmk, fluoromethyl ketone; PARP, poly(ADP-ribose) polymerase; MTS, methanethiosulfonate; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrasodium (acetoxymethyl ester); TMRE, tetramethylrhodamine ethyl ester perchlorate; Mn-SOD, manganese superoxide dismutase.
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probe (sc-803), VDAC (sc-8828), ANT (sc-11433), and Bax (sc-493) were from Santa Cruz Biotechnology. Antibodies against caspase-9 (9501, 9502), caspase-7 (9491, 9491), caspase-3 (9662, 9664), PARP (9541, 9542), hexokinase (C35C4), Cox4 (48445), GAPDH (14C10), were from Cell Signaling Technology. Antibody against FLAG epitope (SAB4200071) was from Sigma. Antibody against LAMP2 was purchased from Invitrogen. Antibody against Trap was obtained from Cell Signaling Technology. Antibody against LAMP2 was purchased from Invitrogen. Antibody against Trap was obtained from Cell Signaling Technology.

**Plasmid and siRNA Transfection**—Plasmids were transfected in 293T and HeLa cells with Lipofectamine 2000 (Invitrogen), and siRNA was transfected in 293T and MA104 cells with siPORT-NeoFX (Ambion) according to the manufacturer’s instructions. Custom-synthetic siRNA against NSP4 was obtained from Dharmacon. Bax siRNA (Flexi Tube Gene Solution for Bax, GS581) was obtained from Qiagen.

**Western Blot Analysis**—Whole cell lysates (extracted with Totex buffer (20 mM Hepes, pH 7.9, 0.35 mM NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 50 mM NaF, and 0.3 mM Na3VO4) containing a mixture of protease and phosphatase inhibitor (Sigma)), cytoplasmic or mitochondrial extract, *in vitro*-translated product, or immunoprecipitated products were prepared and subjected to SDS-PAGE followed by immunoblotting according to standard protocols (19) using specific primary antibody with the manufacturer’s recommended dilutions. For anti-NSP4 antibody, a 1:3000 dilution was used. Primary antibodies were identified with HRP-conjugated secondary antibody (Pierce) and chemiluminescent substrate (Millipore, Billerica, MA). *In vitro*-translated product containing biotinylated proteins were detected by immunoblotting using high sensitivity streptavidin-HRP (Pierce) (supplemental Fig. 1A). Recombinant proteins were purified on Ni2+–nitrilotriacetic acid magnetic agarose beads under native conditions, and the purity was validated by immunoblot analysis using antibodies against NSP4, VDAC, and ANT (supplemental Fig. 1B).

**In Vitro Mitochondrial Import Assay and Cytochrome c Release Assay**—Overnight starved BALB/c mouse livers were isolated and homogenized followed by fractionation as described previously (22). Purified, functionally active mitochondria were resuspended in MRM-S buffer (250 mM sucrose, 10 mM Hepes, 1 mM ATP, 5 mM succinate, 0.08 mM ADP, and 2 mM K2HPO4, pH 7.4), and 50 μg of mitochondria (by total protein) were incubated with different amounts of purified *in vitro* NSP4 with appropriate control (pcDNA6 IVT product) for 1 h at 30°C. The mitochondria were pelleted (7000 × g for 10 min) and suspended in resuspension buffer (7 mM urea, 2 mM thiourea, 4% CHAPS, 120 mM dithiothreitol (DTT), 2% ampholytes, pH 3–10, and 40 mM Tris-HCl), and extracted protein were immunoblotted with anti-NSP4 antibody. The supernatants were analyzed for cytochrome c release by immunoblotting with anti-cytochrome c antibody.

**Analysis of Mitochondrial Depolarization with TMRE Fluorescence**—Functionally active purified mitochondria were incubated with different amount of IVT NSP4 for 10 min at RT. 1 ml of 50 nM TMRE dye dissolved in MRM-S buffer was added to IVT NSP4-treated mitochondria. After 10 min of incubation at room temperature, TMRE fluorescence was measured in a fluorometer (PTI, fluorescence spectrophotometer) with 490 nm excitation wavelength and 575 nm emission scanning.

**Immunofluorescence Microscopy**—To assess localization of NSP4 to mitochondria, HeLa cells were seeded in a 4-well chamber slide (Pharmingen) and transfected with pAcGFP1-C2NSP4, deletion mutants, or fragments of NSP4 cloned in pAcGFP1-C2. After 16 h, cells were fixed with paraformalde-
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hyde (4% (w/v) in (PBS)) for 10 min at room temperature (RT) and then permeabilized with 0.1% Triton X-100 for 20 min at 4 °C. Cell were then incubated with blocking solution (PBS supplemented with 5% (v/v) horse serum and 5% (v/v) goat serum) for 1 h at room temperature followed by a cold PBS (three times) wash and incubation with primary antibodies (α MnSOD) for 2 h at RT. Unbound primary antibodies were washed with PBS (three times) followed by 1 h of incubation (RT) with Rhodamine Red X-conjugated anti-rabbit antibody. After washing five times with PBS, slides were mounted with Vectashield-DAPI and observed under a fluorescence microscope. Excitation and emission detection for each fluor was performed sequentially to avoid cross-talk.

Confocal Microscopy and Imaging—To assay subcellular localization of NSP4 in different organelles such as endoplasmic reticulum, mitochondria, or lysosomes, HeLa cells were processed as described previously and incubated with primary antibodies against Trap α (ER marker), Cox4 (mitochondrial marker), and LAMP2 (lysosome marker) for 2 h at RT. Unbound primary antibodies were washed with PBS (three times) followed by a 1-h incubation (RT) with Alexa 546-conjugated secondary antibody. After washing five times in PBS, confocal microscopy was performed utilizing LSM510-Meta confocal microscopy system (Zeiss) equipped with an argon ion laser (for GFP excitation with the 488 nm line) and a He-Ne laser (for TMRE and Alexa Fluor 546 excitation with the 543 line). A 63 × 1.4 NA oil immersion objective was used for all imaging. For comparisons between multiple samples, images were collected during a single session using identical excitation and detection settings. The detector gain settings were chosen to allow imaging of the desired cells within the linear range of the photomultiplier tube without saturating pixels. For imaging localization of different proteins, randomly chosen fields of cells were imaged with the above laser lines. The complete set of experiments was performed twice to eliminate artifacts arising from individual experiments.

FACS Characterization of Mitochondrial Membrane Potential—At the indicated time points, transfected 293T cells and infected MA104 cells were trypsinized and resuspended in 1 ml of PBS. Suspension was centrifuged at 300 × g for 5 min, and pellets were resuspended into 1 ml of 100 mM TMRE for 20 min at 37 °C before direct analysis on a flow cytometer (Aria II, BD Biosciences) using a 488-nm laser. As a positive control for membrane depolarization, cells were treated with carbonyl cyanide p-chlorophenylhydrazide and stained with TMRE (data not shown).

TUNEL Assay—pcDNSP4-transfected or control vector-transfected 293T cells with or without different treatment were harvested at the indicated time points and stained using a APO-BRDU™ kit (Pharmingen) for terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay according to the manufacturer’s protocol. For flow cytometry, data acquisition and analysis were performed on FACS Aria cytometer using a FACS Diva data management system (BD Biosciences).

Trypsin Treatment of Isolated Mitochondria—To identify proteins located peripherally on the outer mitochondrial membrane, mitochondria fractionated from NSP4-expressing 293T cells (24 h post-transfection) or mouse liver mitochondria were incubated with IVT NSP4 (2 μM) and treated with trypsin on ice for 30 min followed by centrifugation at 6700 × g for 15 min at 4 °C (23). The pellet was washed twice with MES buffer (20 mM Hepes-NaOH, pH 7.4, 220 mM mannitol, 70 mM sucrose, and 0.1 mM EDTA) and analyzed with 15% SDS-PAGE. Western blot analysis was performed using hexokinase- (24) and VDAC (25)-specific antibody as markers of peripheral outer mitochondrial membrane protein and a protein located within the mitochondria, respectively.

Alkaline Treatment of Isolated Mitochondria—To distinguish integral membrane protein from peripheral membrane and soluble proteins, either mitochondria fractionated from pcDNSP4-transfected 293T cells after 24 h or mouse liver mitochondria incubated with IVT NSP4 (2 μM) as before were treated with 0.1 M sodium carbonate (Na2CO3), pH 11.3, for 30 min on ice followed by centrifugation at 13,000 × g for 10 min at 4 °C as described previously (26). The pellet fraction containing the inner and outer mitochondrial membrane were directly solubilized in SDS sample buffer, and the supernatants containing peripheral membrane, intramembrane space, and matrix protein were first concentrated by trichloroacetic precipitation and then solubilized and separated by 15% SDS-PAGE. Western blot analyses was done using hexokinase and VDAC as markers of peripheral outer mitochondrial membrane protein and a protein located within the mitochondria, respectively.

Potassium Chloride (KCl) Treatment of Isolated Mitochondria—To separate the outer and inner mitochondrial membrane either mitochondria fractionated from pcDNSP4-transfected 293T cells after 24 h or mouse liver mitochondria incubated with IVT NSP4 (2 μM) as before were treated with 10 mM KCl for 10 min on ice, as described previously (27), followed by centrifugation at 2500 × g. The pellet fraction contains the intact inner mitochondrial membrane and matrix, leaving the outer mitochondrial membrane and intermembrane space in the supernatants. The pellet fraction was washed and centrifuged at 300 × g before solubilization in SDS sample buffer. The supernatants were first concentrated by trichloroacetic precipitation and then solubilized in SDS sample buffer followed by separation with SDS-PAGE and Western blot analyses using VDAC and Cox4 (28) as markers of outer mitochondrial membrane protein and inner mitochondrial membrane protein, respectively.

Determination of Intracellular Ca2+—293T cells were transfected with pcDNSP4 either in the presence or absence of BAPTA-AM (50 μM) (added 6 h post-transfection), and intracellular Ca2+ concentration was measured as described previously (29, 30) with FURA 2/AM at the indicated time points.

Fluorescence Resonance Energy Transfer (FRET)—To assess localization of NSP4 to mitochondria, HeLa cells were seeded in a four-well chamber slide (Pharmingen) and transfected with pAcGFP1-C2NSP4. After 16 h, cells were treated with 150 mM TMRE for 20 min at room temperature followed by a PBS wash (three times) for 5 min each. Fluorescence microscopy was performed utilizing the LSM710-NLO microscopy system (Zeiss) equipped with an argon ion laser (for GFP excitation with the 488 nm line) and an He-Ne laser (for TMRE excitation with the 543 nm line). A 63 × 1.4 NA oil immersion objective was used.
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for all imaging. For comparisons between multiple samples, images were collected during a single session using identical excitation and detection settings. The detector gain settings were chosen to allow imaging of the desired cells within the linear range of the photomultiplier tube without saturating pixels. For imaging proximity between mitochondria and NSP4, randomly chosen fields of cells were imaged with the above laser lines. Three dishes were imaged for each set of transfections, and the complete set of experiments was performed twice to eliminate artifacts arising from individual experiments. For FRET analyses, defined regions of interest were photobleached at full laser power (100% power and 100% transmission) of the 543-nm laser beam; the change in fluorescence was monitored in both the channels by scanning the whole cell at low laser power (10% power and 0.3% transmission) as described previously (31). Two images were recorded of the donor and acceptor before and after photobleaching. The fluorescence intensities of the two channels were plotted over time as described previously (32).

Statistical Analysis—Data were expressed as means ± S.D. of at least three independent experiments (n ≥3). In all tests, p = 0.05 was considered statistically significant.

RESULTS

NSP4 Induces Ca2+ Ion and Bax-independent Apoptosis—Purified NSP4 protein has been reported to cause diarrhea in mice (5). To characterize its cytotoxic effect, we transiently transfected 293T cells with either pcDNSP4 or empty vector, and after 24 h, apoptosis was measured by measuring DNA fragmentation using TUNEL assay according to the manufacturer's protocol. Expression of the glycosylated (26 and 28 kDa) and nonglycosylated (20 kDa) form of NSP4 was confirmed by immunoblotting with NSP4 antisera (Fig. 1A). Ectopic expression levels of NSP4 protein in pcDNSP4-expressing cells (24 h) and cells infected with SA11 (11–14 hpi) were assessed by Western blotting followed by densitometry analysis by Quantity One software version 4.6.3 (Bio-Rad) using GAPDH as normalization control (Fig. 1B). Results revealed a significant increase in TUNEL-positive cells in pcDNSP4-transfected cells (44%) compared with empty vector (7.4%) (Fig. 1C, I and II). To know whether the apoptosis induced by NSP4 expression was by the extrinsic or intrinsic pathway, cleavage of caspase-8, caspase-9, caspase-7, caspase-3, and PARP was analyzed by immunoblotting and after 24 h, apoptosis was measured by TUNEL assay, which revealed a 25–50% decrease in apoptosis in NSP4-expressing cells treated with Bax-siRNA (30.3%) (Fig. 1C, VI) or BAPTA-AM (24.4%) (Fig. 1C, IV) alone or together (21.5%) (Fig. 1C, VIII) compared with NSP4-expressing cells. Modulation of Ca2+ ion concentration and Bax expression by BAPTA-AM and Bax-siRNA was measured by FURA-2 fluorescence and immunoblotting, respectively. As shown in Fig. 1E, NSP4-mediated elevation of Ca2+ ion concentration was buffered in the presence of BAPTA-AM. Similarly, cellular Bax was significantly reduced (more than 80%) in Bax-siRNA-transfected cells (Fig. 1F). Because Bax-siRNA and Ca2+ chelator could not reverse NSP4-induced apoptosis completely, it suggested a role of other factors in NSP4-mediated apoptosis.

NSP4 Depolarizes Mitochondria and Induces Apoptosis through Intrinsic Pathway—Caspase-9 cleavage without caspase-8 activation indicated involvement of the intrinsic pathway (Fig. 1D), and thus the effect of NSP4 on mitochondrial membrane potential was measured. 293T cells transfected with pcDNSP4 or control vector (16 h post-transfection) were treated with TMRE (100 nM) for 20 min at 37 °C followed by flow cytometric analysis. An increase in mitochondrial depolarization in pcDNSP4-transfected cells (41%) was observed (Fig. 2A, II), compared with controls (5.5%) (Fig. 2A, I). This suggested that NSP4 alone can depolarize mitochondria, independent of viral replication or expression of other viral proteins. To nullify the effects of Bax or elevated Ca2+ ion concentration, the same experiment was repeated in the presence of either Bax-siRNA (Fig. 2A, III and IV) or Ca2+ chelator (Fig. 2A, V and VI) or both (Fig. 2A, VII and VIII), as described previously. Concurrent with previous results, the presence of Ca2+ chelator (23.9%) and Bax-siRNA (29%) resulted in decreased depolarization compared with only NSP4-expressing cells, but a still significant amount of mitochondrial membrane potential dissipation (20%) was observed compared with 293T control cells in the presence of both Bax-siRNA and Ca2+ chelators (Fig. 2A). To further analyze whether this depolarizing effect of NSP4 is direct or dependent on other cellular factors, increasing amounts of purified IVT NSP4 was incubated with purified functional mouse liver mitochondria. Mitochondrial membrane potential was measured after incubation with TMRE (50 nm) as described under “Experimental Procedures.” Results confirmed that purified IVT NSP4 can depolarize mitochondria in a cell-free in vitro system (Fig. 2B), suggesting that NSP4 can depolarize mitochondria both in cellular and in cell-free conditions, independent of other viral proteins and cellular factors. To see the downstream effect of mitochondria depolarization, 293T cells, transiently transfected with pcDNSP4 or empty vector control, were either treated with BAPTA-AM and Bax-siRNA or left untreated. After 24 h, the presence of cyt c in cytosol was assessed by immunoblotting. As shown in Fig. 2C, cyt c release was observed in pcDNSP4-transfected cells, and in the presence of BAPTA-AM and Bax-siRNA, release of cyt c was attenuated, but a still significant amount of cyt c was found in the cytosol. To verify further, cyt c release assay was done with purified mouse liver mitochondria and IVT NSP4 protein. Consistent with previous results, concentration-dependent cyt c release from mitochondria was observed in the presence of purified NSP4 in a cell-free system (Fig. 2D) suggesting that NSP4-induced apoptosis may be triggered by a direct effect on
mitochondria. To confirm this, pcDNSP4 or empty vector-transfected 293T cells were treated with broad spectrum caspase inhibitor Z-VAD-fmk (10 \(\mu M\)), and the release of cytochrome c from mitochondria was assessed by immunoblotting (Fig. 2E). As shown in Fig. 2E, release of cytochrome c from mitochondria to cytosol was observed in the presence or absence of caspase inhibitor suggesting that caspase activation by NSP4 is downstream of cytochrome c release. However, caspases play a vital role in NSP4-induced cell death, as apoptosis is significantly inhibited in the presence of Z-VAD-fmk (10 \(\mu M\)) (Fig. 2E).

NSP4 Localizes to Mitochondria—Because previous results suggest the direct effect of NSP4 on mitochondria, it was hypothesized that NSP4 may translocate to mitochondria. To examine this possibility, MA104 cells were either infected with the SA11 strain (2 m.o.i.) or mock-infected before mitochondria-enriched fractions were isolated at increasing time points. Immunoblotting with NSP4 antisera revealed the presence of nonglycosylated NSP4 (20 kDa) in mitochondrial fractions from 4 hpi (Fig. 3A). Similarly, mitochondrion-enriched fractions of pcDNSP4-transfected 293T cells for 24 h revealed the presence of nonglycosylated NSP4 (20 kDa), suggesting that mitochondrial translocation of NSP4 was independent of other viral components (Fig. 3B). The mitochondrial fraction was immunoblotted with Cox4-specific antibody as mitochondrial protein control and anti-calnexin, anti-lysosomal-associated membrane protein 2 (LAMP2), for detecting other organelar contamination. As shown in Fig. 3B, lysosomal contamination was not observed, although minimal ER contamination was found. To eliminate this ER contamination, subcellular fractionation by gradient centrifugation using iodixanol was car-
ried out, and copurification of NSP4 with mitochondria was confirmed (supplemental Fig. 3A). To confirm whether mitochondrial translocating ability of NSP4 is independent of other cellular factors, purified functional mouse liver mitochondria were incubated with IVT NSP4. Mitochondrial fraction was precipitated and subjected to immunoblotting. Results revealed the presence of NSP4 with the mitochondrial proteins (Fig. 3C). The proximity of mitochondria and NSP4 was further confirmed by FRET. There was a significant overlap between the emission spectra of GFP and excitation spectra of TMRE making it a well matched FRET pair. When two FRET partners remained closer than 10 nm, the intensity of fluorescence emission of the donor (GFP) was quenched by the acceptor (TMRE). This can be detected by photobleaching of the acceptor fluor, which results in an increase of donor emission intensity. To visualize the spatial proximity, we transfected pAcGFP1-C2NSP4 in HeLa cells, and after 16 h, the cells were labeled with TMRE (150 nm). To see whether TMRE quenches the fluorescence of NSP4-GFP, photobleaching of TMRE was done as described under "Experimental Procedures," which showed an immediate increase in emission of NSP4-GFP indicating close proximity of two fluors (Fig. 3, D and E). Overall, the results confirmed the presence of NSP4 protein in mitochondria. Previously it was reported that NSP4 translocates to the endoplasmic reticulum. To confirm its other subcellular localization, we carried out confocal microscopy with mitochondria, endoplasmic reticulum, and lysosomal markers. Results confirmed the localization of NSP4 in mitochondria and endoplasmic reticu-
but not in the lysosome (supplemental Fig. 3B). The proportion of NSP4 present in mitochondria and endoplasmic reticulum was also confirmed by subcellular fractionation of SA11-infected MA104 cells (8 hpi) using sucrose gradient as described under “Experimental Procedures.” Results showed that at 8 hpi, the amount of NSP4 present in endoplasmic reticulum was 3-fold higher than that in mitochondria (supplemental Fig. 3C).

61–83 Amino Acids of NSP4 Comprise the MTS—To map the MTS region, we generated a panel of sequential N- and C-terminal truncated mutants of NSP4 cloned in pAcGFP1-C2 (Fig. 4A). These constructs were designed to encompass or omit potential α-helices (Fig. 4D). To know the subcellular localization of the mutant NSP4 compared with the wild type, HeLa cells were transfected with either pAcGFP1-C2 or wild type or NSP4 mutant constructs, and after 16 h the cells were fixed, and α-MnSOD (mitochondrial marker) was stained using anti-α-MnSOD antibody followed by anti-rabbit rhodamine-labeled secondary antibody. When pAcGFP1-C2 was expressed alone in HeLa cells, a diffuse green fluorescence was observed in both the cytoplasm and nucleus, but in the case of NSP4-GFP fusion protein, fluorescence was found to localize at filamentous cytoplasmic structures, confirmed as mitochondria by indirect immunofluorescence with α-MnSOD (red) (Fig. 4B). The N-terminal truncated NSP4 mutant, NSP4Δ1–83 only showed diffused fluorescence and no colocalization with the mitochondrial marker, whereas other N-terminal truncated mutants, NSP4Δ1–25, NSP4Δ1–48, and NSP4Δ1–60, showed mitochondrial localization similar to wild type NSP4 (Fig. 4B). In the case of C-terminal truncated mutants, except for NSP4Δ74–
which showed diffused fluorescence-like control vector (pAcGFP1-C2), the vector of all other mutants, NSP4/H9004
143–175 and NSP4/H9004 92–175, showed mitochondrial localization (Fig. 4B). Based on these results, the 61–91-amino acid stretch can be predicted to harbor the MTS region. Sequence analysis revealed the presence of one amphipathic α-helix within this region (Fig. 4C). The presence of amphipathic α-helices has been reported in other mitochondrial translocating cellular and viral proteins (33–34). But to confirm whether this helix alone comprises the MTS or flanking regions are also necessary, different small fractions within the 61–91-amino acid region were cloned, and subcellular localization was observed as described earlier NSP4/61–91 and NSP4/61–83 mutant showed mitochondrial localization like wild type NSP4 but NSP4/74–91 showed diffused fluorescence like empty vector (Fig. 4B). This result revealed that not only the helical region (74–83) within the 61–83-amino acid region but the flanking region (amino acids 61–73) was also required for mitochondrial localization. NSP4 Integrates Both to Outer and Inner Mitochondrial Membrane—After confirming that NSP4 localizes to mitochondria, proper localization of NSP4 within mitochondria was examined. To determine whether NSP4 remains exposed (completely or partially) on the surface of outer mitochondrial membrane or is integrated within the mitochondria, we treated the mitochondrial fraction isolated from either pcDNSP4 transiently transfected 293T cells or from IVT NSP4-treated mouse liver mitochondria with increasing concentrations of trypsin. Western blot analysis was performed with antibodies against NSP4, hexokinase as a peripheral protein marker, and VDAC as an integral protein marker. Results showed that trypsin completely cleaved hexokinase but not VDAC and NSP4, suggesting that NSP4 was not exposed to the surface of the mitochondria (Fig. 5A, top). To rule out the possibility that the procedure of mitochondria isolation may affect trypsin sensitivity of NSP4, experiments were repeated in the presence of 0.1% Triton X-100. As expected, both VDAC and NSP4 were cleaved by

FIGURE 4. Identification of mitochondrial translocation sequence of NSP4. A, schematic representation of N- and C-terminal NSP4 mutants generated in pACGFP1-c2 vector as described under “Experimental Procedures.” B, 60–83 amino acids is the shortest region of NSP4 that acts as MTS. HeLa cells were transfected with either empty GFP-expressing vector or wild type NSP4 or mutant constructs of NSP4 as GFP fusion protein (green). After 16 h of transfection, cells were fixed and stained with anti-α-MnSOD antibody (mitochondrial marker) followed by rhodamine-labeled (red) secondary antibody and DAPI (blue). The cells were visualized with a fluorescence microscope to assess localization of NSP4 (green) and mitochondria (red). C, helical wheel representation (helical wheel projection; Don Armstrong) of a predicted helix formed by residues 74–83 of NSP4. Hydrophilic residues are shown as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons. The most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue. D, putative helix prediction (H helix) within NSP4 sequence using PSIPRED (PSIPRED Server) and sspro (Phyre Server) programs.
trypsin in the presence of Triton X-100 confirming that NSP4 is integrated within the mitochondria (Fig. 5A, bottom).

For further confirmation, isolated mitochondria were treated to separate the integral membrane proteins from peripheral membrane and soluble proteins in the intermembrane space and matrix with sodium carbonate, pH 11.5, followed by differential centrifugation. Immunoblotting was performed with anti-NSP4 antibody, and hexokinase and VDAC were used as peripheral and integral protein markers, respectively. As shown in Fig. 5B, NSP4 was confirmed to be a membrane integral protein. Furthermore, isolated mitochondria were treated with 10 mM KCl followed by differential centrifugation to separate inner membrane from outer membrane and intermembrane space, as KCl treatment disrupts the outer membrane while leaving the inner membrane intact. Fractions were subjected to Western blot analysis with anti-NSP4 antibody, and Cox4 and VDAC proteins were assessed as inner and outer membrane markers, respectively. The result revealed the presence of NSP4 in both outer and inner membrane fractions (Fig. 5C).

**NSP4 Interacts with VDAC and ANT**—Different mitochondrial proteins play an essential role in controlling apoptosis (35). Because NSP4 was found to be a mitochondrial integral protein, it is possible that it interacts with VDAC and ANT, which are targeted by several other viral mitochondrion-translocating proteins (28, 36–38). Hence, to know the functional involvement of VDAC and ANT, these were cloned in pcDNA6 and transfected in 293T cells. After 24 h, cells were lysed, and subcellular fractions were separated as described previously. Immunoblotting revealed that both overexpressed VDAC and ANT were present in mitochondria in transfected cells (Fig. 6A), although small amounts of VDAC were observed in the cytoplasm too (Fig. 6A). To detect interaction between NSP4 and VDAC and ANT, we either cotransfected pcDVEDAC1 and pcDANT3 with pFLAG-CMV6-NSP4, or these three constructs were transfected individually. After 24 h post-transfection, immunoprecipitation was done (with either anti-FLAG antibody or anti-His antibody) as mentioned under “Experimental Procedures,” followed by immunoblotting with the reciprocal antibody. Irrespective of whether NSP4 was pulled

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**FIGURE 5.** **NSP4 is present in both outer and inner membrane of mitochondria.** A, trypsin sensitivity assays reveal the presence of NSP4 on integral membrane. Either mitochondrion-enriched fraction isolated after 24 h from 293T cells transfected with pcDNSP4 (left) or pelleted rat liver mitochondria after being incubated with IVT NSP4 (right) were treated with trypsin (25–50 μg/ml) in the presence or absence of Triton X-100. Immunoblot analysis for NSP4, hexokinase (marker for peripheral membrane), and VDAC (integral membrane marker) revealed no effect of trypsin on NSP4 and VDAC in the absence of Triton X-100 (top panel), whereas in the presence of Triton X-100, both NSP4 and VDAC were degraded. Peripheral membrane marker protein hexokinase is degraded both in the presence or absence of Triton X-100. B, alkaline treatment confirmed presence of NSP4 on both inner and outer mitochondrial integral membrane. Mitochondrion-enriched fraction isolated after 24 h from 293T cells transfected with pcDNSP4 (left) or pelleted mouse liver mitochondria after being incubated with IVT NSP4 (right) were subjected to alkaline treatment (NaHCO₃). Reaction mixture was centrifuged to pellet down mitochondrial integral membrane. Both supernatants (Supe) and pellets were immunoblotted using NSP4 antisera, anti-VDAC, and anti-hexokinase antibodies. C, KCl treatment confirms copurification of NSP4 with both mitochondrial outer and inner membrane integrated proteins. As described previously (B) mitochondrion-enriched fractions were subjected to KCl treatment. Treated fractions were fractionated by differential centrifugation followed by immunoblotting with NSP4 antisera, anti-VDAC, and anti-Cox4 antibody.
down using anti-FLAG or VDAC1/ANT3 by anti-His antibodies, specific interaction between NSP4 and VDAC1 and ANT3 was confirmed (Fig. 6B). Input lysates were probed using Cox4, anti-FLAG, and anti-His antibodies. To confirm this interaction during virus infection, 293T cells expressing either VDAC1 or ANT3 were either infected with SA11 (2 m.o.i.) or mock-infected. Coimmunoprecipitation was done with anti-NSP4 antibody after 10 hpi, followed by immunoblotting with anti-His and anti-His antibodies. Results confirmed interaction between NSP4 and VDAC1 and ANT3 protein during virus infection (Fig. 6C). Thus, it can be concluded that NSP4 causes depolarization of mitochondria and release of cyt c probably through interacting with VDAC and ANT.

During SA11 Infection Ca$^{2+}$ Ion Elevation and Bax Activation Independent Mitochondrial Depolarization Occurs—From the previous experiments, it was confirmed that overexpression of NSP4 leads to its translocation to mitochondria (Fig. 3B) and dissipation of mitochondrial membrane potential (Fig. 2B), but whether similar effects are observed during virus infection was not proved. Therefore, we either infected MA104 cells with SA11 at 2 m.o.i. or mock-infected. After 2–14 hpi, cells were incubated with TMRE (100 nM) for 20 min, and depolarization of mitochondria was measured by flow cytometry. Results revealed a significant increase in depolarization (4.6–66.4%) in a time-dependent manner from 5 h post-infection (Fig. 7A). To confirm whether this depolarization was independent of Ca$^{2+}$/H$^{+}$ ion flux and Bax activation, cells were transiently transfected with Bax-siRNA. After 24 h, cells were infected with SA11 (2 m.o.i.), and after 2 hpi 50 μM BAPTA-AM was added and mitochondrial depolarization was measured at 2–14 hpi. As shown in Fig. 7B, BAPTA-AM and Bax-siRNA together reduced the intensity of mitochondrial depolarization (32.4% at 14 hpi) compared with virus-infected cells (66.4% at 14 hpi) (Fig. 7A), although still significant mitochondrial depolarization was observed, which is independent of Ca$^{2+}$ ion or Bax activation. This is consistent with our previous results following NSP4 overexpression (Fig. 2A). To confirm this depolarization is due to NSP4, we infected NSP4 siRNA or scrambled NSP4 siRNA-transfected MA104 cells and measured the mitochondrial depolarization. Compared with scrambled NSP4
siRNA-transfected cells, a significant decrease in mitochondrial depolarization was observed in NSP4 siRNA-transfected cells following SA11 infection (2–14 hpi) (Fig. 7C).

At 5 hpi, mitochondrial depolarization was observed in SA11-infected MA104 cells, suggesting release of cyt c from the mitochondria into the cytosol. To assess this, MA104 cells were either infected with SA11 at 2 m.o.i. or left untreated for given time points, and Western blot analysis was done for cytoplasmic cyt c following subcellular fractionation. As expected, release of cyt c from mitochondria was detected as early as 5 hpi, whereas Bax was found to translocate to mitochondria from 11 hpi onward (Fig. 8A). C, SA11-induced dissipation of mitochondrial membrane depolarization inhibited by NSP4 siRNA. MA104 cells were either infected with SA11 at 2 m.o.i. or mock-infected after 24 h transfection with either NSP4 siRNA or scrambled NSP4 siRNA. After 2–14 hpi cells were incubated with TMRE (100 nm) for 30 min at 37 °C, and TMRE accumulation was analyzed using flow cytometry. Representative bar diagrams showed cells with depolarized mitochondria.

In Rotavirus Early Infection, Intrinsic Apoptotic Stimuli Initiated by NSP4 Is Suppressed by Virus-initiated Survival Pathways—Cyt c release into cytoplasm has been shown to facilitate activation of caspase-9 by formation of functional apoptosome (39). Thus activation of caspases or other apoptotic marker proteins was analyzed in a time-dependent manner following SA11 infection. Cell lysates were prepared from MA104 cells infected with SA11 or mock-infected (2–14 hpi) followed by immunoblotting with caspase-9-, PARP-, pAKT-, AKT-, and XIAP-specific antibodies. Up-regulation of anti-apoptotic protein XIAP and AKT phosphorylation was observed as early as 2 hpi until 8 hpi (Fig. 8D), which is consistent with our previous report (40). In contrast, cleavage of caspase-9 and PARP protein was observed after 11 hpi (Fig. 8D); previous studies with rotaviral strain A5-13 from our laboratory have reported induction of apoptosis during late hours of infection (after 12 hpi), which was attributed to induction of the anti-apoptotic pathway during early infection (40). Thus, to confirm whether activation of PI3K/AKT pathway counteracts the effect of cyt c release, MA104 cells were either infected with...
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**FIGURE 8.** NSP4-mediated apoptotic signaling is counteracted by virus-involved pro-survival pathways. A, correlation of cyt c release into cytosol with Bax translocation to mitochondria during rotavirus (SA11) infection. At 0 to 14 hpi, MA104 cells infected with SA11 (2 m.o.i.) were harvested and subjected to subcellular fractionation. Western blot analysis was done on cytosolic and mitochondrial fraction using anti-cyt c and anti-Bax antibodies. Cox4 and GAPDH were used as loading control for mitochondrial and cytosolic fractions, respectively. B, down-regulation of NSP4 by siRNA delays cyt c release into cytosol. MA104 cells were treated with NSP4 siRNA or scrambled NSP4 siRNA for 24 h followed by infection with SA11 at 2 m.o.i. At increasing time point (0–14 hpi) cells were subjected to subcellular fractionation. Cytosolic extracts were analyzed for cyt c by immunoblotting. GAPDH was used as protein loading control. C, NSP4 siRNA down-regulates NSP4 during infection. MA104 cells were transfected with NSP4 siRNA or control (CON) siRNA for 24 h followed by infection with SA11 at 2 m.o.i., and after 8 hpi whole cell lysates were immunoblotted with NSP4 antisera. GAPDH was used as protein loading control. D, rotavirus (SA11) infection activates both apoptotic and anti-apoptotic proteins. MA104 cells were infected with SA11 at 2 m.o.i., and cells were harvested at increasing time points during 0–14 hpi. Caspase-9, PARP, pAKT, AKT, and XIAP proteins were analyzed by Western blot analysis. GAPDH was used to confirm equal protein loading. E, inhibition of rotavirus induced prosurvival pathway induces early apoptosis; MA104 cells were infected with SA11 at 2 m.o.i., and after virus absorption, PI3K inhibitor LY294002 (10 μM) was added to media. At increasing time points, cells were harvested, and pAKT, AKT, XIAP, caspase-9, PARP, and GAPDH were analyzed by immunoblotting. F, down-regulation of NSP4 counteracts early apoptosis initiation by LY294002 during rotavirus infection. MA104 cells were transfected with NSP4 siRNA for 24 h followed by infection with SA11 at 2 m.o.i. After virus absorption, cell were treated with 10 μM of LY294002 and harvested at increasing time points (0–14 hpi). Caspase-9 cleavage was assessed by immunoblotting.

SA11 or mock-infected and treated with 10 μM LY294002 (PI3K inhibitor) post-absorption. Caspase-9 cleavage and expression of pAKT and XIAP were measured by immunoblotting in a time-dependent manner. As shown in Fig. 8E, LY294002 treatment resulted in down-regulation of XIAP protein and early activation of caspase-9 cleavage. In addition, down-regulation of both NSP4 (NSP4 siRNA) and PI3K/AKT (by LY294002) significantly delayed caspase-9 cleavage until 11–14 hpi (Fig. 8F) compared with only LY294002-treated cells (Fig. 8E). For further confirmation of the role of the NSP1-activated PI3K/AKT pathway, we infected MA104 cells with NSP1 mutant rotavirus strain A5-16 at 2 m.o.i. and analyzed the cyt c release from mitochondria and caspase activation at the indicated time points (Fig. 9A). Results revealed that compared with SA11 infection (Fig. 8A), there was no change in cyt c release during A5-16 infection (Fig. 9A), but caspase activation starts at an earlier time point (8 hpi) (Fig. 9A). This suggested the role of the PI3K/AKT pathway in preventing NSP4-initiated apoptotic stimuli during early infection. This counter-acting role of NSP1 was further proved when release of cyt c from mitochondria (Fig. 9C) and cell death was measured (Fig. 9D) in NSP4 and NSP1 cotransfected cells. Compared with only NSP4-expressing cells, significant decrease in apoptosis was observed in NSP4- and NSP1-coexpressing cells. Overall results confirmed the role of NSP4 in early release of cyt c from mitochondria to cytosol, but a delay in caspase-9 activation was due to the activation of the cellular survival pathway during rotavirus infection.

**DISCUSSION**

Rotaviral nonstructural protein NSP4 is the first discovered viral enterotoxin (3–5) with subcellular localization at ER (10). In this study, we showed NSP4 localizes to the mitochondria and induces apoptosis. Other viral proteins such as PB1F2 of influenza virus (36), HBX of hepatitis B virus (28), and VPR of human immune deficiency virus (37) localize to mitochondria...
and trigger apoptotic pathways. Induction of apoptosis during virus infection is important for viral release and dissemination of viral progeny (41). Unlike influenza virus (36), human immunodeficiency virus (37), HTLV1 (38), etc., no rotavirus-encoded protein has been shown to directly induce apoptosis. Previous studies have analyzed an increase in Ca$^{2+}$ ion concentration (12) and activation of proapoptotic protein Bax (13) as a probable cause of rotavirus-induced apoptosis. Ca$^{2+}$ homeostasis is stringently maintained in cells because disruption in Ca$^{2+}$ homeostasis predisposes cells toward apoptosis (42). Bax is a Bcl2 family cytoplasmic protein, which after activation by diverse stimuli inserts into mitochondria leading to mitochondrial depolarization and release of pro-apoptotic proteins from mitochondria resulting in initiation of caspase activation and apoptosis (43–47).

In this study, we propose that NSP4-mediated apoptosis is not fully dependent on Ca$^{2+}$ ion concentration or Bax activation, because significant apoptosis was observed in NSP4-expressing cells in the presence of Bax-siRNA and Ca$^{2+}$ chelator (Fig. 1C). This suggested that some other proapoptotic factor or pathways are activated by NSP4. Apoptosis can be broadly divided into extrinsic and intrinsic pathways. Extrinsic pathway is initiated by death receptor stimulation and caspase-8 activation, and the intrinsic pathway involves mitochondrial dysfunction resulting in release of proapoptotic proteins from mitochondria and activation of caspase-9 (48–50). Analysis of both pathways following NSP4 overexpression revealed depolarization of mitochondria, release of cyt c into cytosol, and activation of caspase-9 both in vitro (Figs. 2, A and C, and 1D) in a cell-free condition (Fig. 2, B and D) and during virus infection (Fig. 7A, and Fig. 8, A and D), suggesting the involvement of intrinsic pathway. Because pcDNSP4 expression or IVT-NSP4 treatment resulted in cyt c release from mitochondria and NSP4 siRNA significantly delayed cyt c release in SA11 infection (Fig. 8B), a direct role of NSP4 on mitochondria was postulated. Close proximal presence of NSP4 and mitochondria was observed by doing FRET analysis suggesting that NSP4 may have to translocate to mitochondria for this function (Fig. 3D).

The presence of NSP4 in mitochondria was further confirmed by immunoblotting subcellular fractionation of cells infected with SA11 or transfected with pcDNSP4 (Fig. 3, A and B). Quantitation of NSP4 in subcellular fractions confirmed 3-fold higher concentration of NSP4 in ER compared with the mitochondrial fraction (supplemental Fig. 3b).

During NSP4 overexpression all forms of NSP4 were evident (Fig. 1A), but only the nonglycosylated form was copurified with the mitochondrial fraction (Fig. 3B), which is further confirmed by translocation of IVT NSP4 to mitochondria (Fig. 3C) as in vitro transcription and translation do not support glycosylation.

To know the shortest region necessary for mitochondrial translocation of NSP4, various deletion mutants were constructed. Immunofluorescence microscopy following transfection identified a 23-amino acid long region (amino acids

![Rotaviral NSP4 Translocates to Mitochondria](image-url)

**FIGURE 9.** Rotavirus-encoded protein NSP1 counteracts proapoptotic signaling of NSP4. A, absence of NSP1 does not affect rotavirus-induced cyt c release but facilitates early apoptosis. MA104 cells were infected with NSP1 mutant strain A5-16 (2 m.o.i.), and at increasing time points (0–14 hpi) cells were harvested. Immunoblot analysis of either cytosolic fraction or whole cell lysate for either cyt c released into cytosol or caspase-9, caspase-3 cleavage was done. GAPDH was used as loading control for cytosolic fraction. B, co-overexpression of NSP4 and NSP1. 293T cells were transfected with either nonexpressing vector control or pcDNSP4 or cotransfected with pcDNSP4 and pcDNSP1 followed by Western blot analyses using NSP1 and NSP4 antisera. β-Actin was used to confirm equal protein loading. C, NSP1 minimized cyt c releasing effect of NSP4. 293T cells were transfected with either nonexpressing vector control or pcDNSP1 or cotransfected with pcDNSP4 and pcDNSP1 followed by subcellular fractionation and Western blot analyses of the cytoplasmic fraction using anti-cyt c antibody. β-Actin was used to confirm equal protein loading. D, NSP1 diminished proapoptotic stimuli elicited by NSP4; 293T cells were transfected with empty vector control or pcDNSP4 or cotransfected with pcDNSP4 and pcDNSP1 and incubated with terminal deoxynucleotidyltransferase and FITC-conjugated anti-BrdU monoclonal antibodies, and DNA fragmentation was measured after 24 h by TUNNEL assay using flow cytometry.
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61–83) to be sufficient for mitochondrial translocation (Fig. 4B). There are other regions involved for more efficient translocation. It was reported that full-length NSP4 is targeted to the ER by an uncleaved signal sequence (amino acids 25–44) (6) and is inserted into the membrane by a viroporin region (amino acids 47–90) (10). Identified MTS(61–83) is within the reported viroporin domain, which is responsible for NSP4-mediated membrane destabilization. Similar to other mitochondrial proteins, NSP4 also has a putative amphipathic helix (amino acids 74–83) (Fig. 4C) within the MTS region (33, 34).

Once the MTS region of NSP4 was identified, the next question was to know the localization of NSP4 within mitochondria. No effect of trypsin on mitochondrial NSP4 in the absence of detergent (Triton X-100) (Fig. 5A, upper panel) and degradation of NSP4 by trypsin in the presence of Triton X-100 (Fig. 5A, lower panel) suggested that NSP4 is not localized on peripheral mitochondrial membrane but is part of integral membrane. Similarly alkaline (sodium carbonate, pH 11.5) and KCl treatment confirmed it to be an insoluble protein, embedded both in the outer and inner mitochondrial membranes (Fig. 5, B and C), which is similar to the PB1F2 protein of influenza virus (32). This suggested that NSP4 might be targeting VDAC and ANT. To confirm this hypothesis, NSP4 was coimmunoprecipitated with VDAC1 and ANT3 in both SA11-infected and pcDNSP4-expressing cells (Fig. 6, B and C). Isoforms VDAC1 and ANT3 were utilized because of their abundance and ubiquitouness (51, 52). Overall, it can be hypothesized that NSP4 contributes to mitochondrial dysfunction by interacting with VDAC and ANT possibly through VDAC closure with impaired substrate supply to mitochondria and or inhibition of ANT with a shortage of ATP production that leads to apoptosis. But whether a single NSP4 molecule binds with both VDAC and ANT forming a bridge or whether it binds separately cannot be defined from this study, and it does not exclude possible interaction of NSP4 with other mitochondrial proteins involved in mitochondrial permeability transition. Further in-depth analysis is required to know the exact mechanism of permeability transition.

During infection, NSP4 was found to be present in mitochondria as early as 4 hpi, which increases with time (Fig. 4A). This correlates with significant mitochondrial depolarization and release of cyt c from 5 hpi (Figs. 7A and 8A). In contrast, Bax translocation was observed at 11 hpi (Fig. 8A) suggesting the two processes are independent. Consistent with our observation, a previous report with a rotavirus strain (RRV) had shown cyt c release at 9 hpi and Bax activation at 12 hpi (13). Cytosolic cyt c interacts with APAF-1(157–44) triggering its oligomerization, which allows the recruitment of procaspase-9 leading to its autocalytic cleavage and activation. Activated caspase-9 then initiates the cascade of caspase activation leading to apoptosis (39, 53, 54). Surprisingly, we observed caspase-9 cleavage after 10–11 hpi despite cyt c release at 5 hpi (Fig. 8D). Thus, some other regulatory pathway may be involved to prevent early activation of caspase-9 by cyt c. Induction of anti-apoptotic pathways such as PI3K/AKT during early steps of virus infection has been reported previously (40). Both rotavirus NSP1 and influenza virus NS1 proteins were shown to interact with PI3K and induce AKT phosphorylation and up-regulation of XIAP (40, 55), which can bind with caspase-9 and caspase-3 and inhibit their autocatalytic activity (56, 57). In SA11 infection, both AKT phosphorylation and XIAP up-regulation were observed as early as 2 hpi but was down-regulated after 10–12 hpi (Fig. 8D). The role of PI3K/AKT activation in delaying apoptosis was confirmed when significant caspase-9 cleavage was observed as early as 8 hpi in the presence of the PI3K inhibitor (Fig. 8E). However, in the presence of both PI3K inhibitor and NSP4 siRNA, caspase-9 activation was delayed further (14 hpi) (Fig. 8F), suggesting that down-regulation of NSP4 results in inhibition of initiation of proapoptotic signaling resulting in delayed apoptosis. Induction of apoptosis during late hours is due to both host innate immune responses and other virus-induced pathways for efficient viral dissemination (40).

Overall, the results from a previous study and our study suggested a dual role of NSP4 during rotavirus infection. It elevates the Ca2+ ion concentration during infection, which results in disturbance of cellular homeostasis, triggering stress response, and plays a vital role in pathogenesis. In addition, we have shown that it translocates to mitochondria and disrupts mitochondrial membrane potential, eliciting an intrinsic apoptotic pathway. But the activation of early apoptosis may not be beneficial for virus as it will abort its replication and formation of infectious progeny (14). To maintain this balance, rotavirus counteracts the effect of NSP4 by activating the PI3K/AKT pathway through the rotavirus protein NSP1. We have previously shown early apoptosis induction and slower growth rate of the NSP1 mutant strain compared with the wild type rotavirus strain (40). NSP1 mutant strain A5-16 resulted in cyt c release (Fig. 9A), and in mitochondrial depolarization (supplemental Fig. 2) similar to SA11 (5 hpi), but unlike SA11 strain, early caspase-9 activation (Fig. 9A) was observed. Counteracting the role of NSP1 was further confirmed when only NSP4-expressing cells induced much higher apoptosis compared with NSP4- and NSP1-coexpressing cells (Fig. 9D), despite comparable cyt c release from mitochondria (Figs. 2C and 9C). This type of cell death regulation by two virus-encoded proteins has been also observed in influenza virus, polio virus, and adenovirus, etc. (40, 57–59). During the course of evolution, viruses have employed virus proteins and host intrinsic apoptotic pathways for their own benefit.

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