Rotavirus Nonstructural Protein 1 Suppresses Virus-Induced Cellular Apoptosis To Facilitate Viral Growth by Activating the Cell Survival Pathways during Early Stages of Infection
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Following virus infection, one of the cellular responses to limit the virus spread is induction of apoptosis. In the present study, we report role of rotavirus nonstructural protein 1 (NSP1) in regulating apoptosis by activating prosurvival pathways such as phosphatidylinositol 3-kinase (PI3K)/Akt and NF-κB (nuclear factor κB) during early hours of infections (2 to 8 hpi). The NSP1 mutant strain A5-16 induces weak and transient activation of Akt (protein kinase B) and p65 NF-κB compared to the isogenic wild-type strain A5-13 in MA104 or HT29 cells. The weak NF-κB promoter activity or Akt phosphorylation after A5-16 infection could be complemented in cells transfected with plasmid expressing NSP1 after infection with the rotavirus A5-16 strain. In cells either infected with A5-13 or transfected with pcD-NSP1, coimmunoprecipitation of NSP1 with phosphoinositide 3-kinase (PI3K) was observed, indicating that strong activation of PI3K/Akt could be due to its interaction with NSP1. In addition, after infection with same multiplicity of infection, A5-16 showed reduced number of viral particles compared to the A5-13 strain at the end of the replication cycle. A lower growth rate could be due to weak induction of PI3K/Akt and NF-κB, since the A5-13 strain also showed reduced growth in the presence of PI3K or NF-κB inhibitors. This effect was interferon independent; however, it was partly due to significantly higher caspase-3 activity, poly-ADP ribose polymerase (PARP) cleavage, and apoptosis during earlier stages of infection with the NSP1 mutant. Thus, our data suggest that NSP1 positively supports rotavirus growth by suppression of premature apoptosis for improved virus growth after infection.

Virus infection results in the activation of a variety of cellular signaling pathways that are required not only for mounting an antiviral response to infection but are also exploited by viruses to support their replication in host cells. All stages of viral infection including entry, the production of double-stranded RNA (dsRNA), and the expression of viral proteins can activate innate immune response (35). Viral infection stimulates the phosphorylation and subsequent dimerization of a ubiquitously expressed 55-kDa protein, IFN regulatory factor 3 (IRF-3), which then translocates to the nucleus and induces type I interferons (IFNs; IFN-α and -β) as the first line of defense against infections (29, 35). The secreted IFNs signal the production and activation of antiviral proteins in neighboring cells to control the spread of infection. To counteract these antiviral responses, viruses have evolved mechanisms to suppress the IFN-mediated signaling pathways. VP35 of Ebola virus, NS1 and NS2 of respiratory syncytial virus (RSV), NS1 of influenza virus, the E6 protein of human papillomavirus, etc., suppress IFN induction by inhibiting either the activation of IRF3 (5, 23, 50, 52) or the IFN-induced JAK/STAT pathway (30). Other than the inhibition of innate immune responses, it is also important for a virus to keep the infected cell alive to complete its life cycle. Thus, viruses have also evolved mechanisms to modulate the host cellular apoptotic pathways. For example, NS1 and NS2 proteins of RSV suppress premature apoptosis of host cell by a nuclear factor κB (NF-κB)-dependent and IFN-independent mechanism (6), whereas poliovirus, influenza virus, and dengue virus have been shown to limit premature cell death by early activation of phosphoinositide 3-kinase (PI3K)/Akt pathway (2, 17, 39).

Rotaviruses, members of the family Reoviridae, are the major cause of severe gastroenteritis in children younger than 5 years of age. Calves, piglets, and other animals of economic importance are also susceptible to rotavirus infection (21). Rotaviruses generally infect the enterocytes of the small intestine; however, there have recently been increasing reports of extraintestinal infections (7), highlighting the importance of better knowledge of the mechanisms of viral pathogenesis and virus-host cell interactions (11, 32).

The virus is a icosahedral structure consisting of three concentric layers of proteins and a genome of 11 dsRNA segments (21). In addition to the six structural proteins (VP1 to VP4, VP6, and VP7) which form the virion, the virus also encodes
six nonstructural proteins (NSP1 to NSP6). Nonstructural proteins (NSPs) are of great interest since these are translated only in host cells after virus infection and do not form part of the mature infectious virus. In general, the NSPs of viruses have been associated with diverse functions such as interactions of virus with host cell, RNA binding, evasion of immune response, inhibition of cellular translation, etc. (2, 6, 8, 17, 23, 30, 50, 52). There are limited reports regarding the role of rotavirus-encoded NSPs. Rotavirus NSP4 is a putative viral enterotoxin (18), NSP3 has been implicated in PABP binding and nuclear translocation of PABP binding protein (27), NSP3 has been also shown to interact with protein kinase R (PKR) (38), and NSP1 has been shown to inhibit the induction of IFN by inducing the degradation of IRF3-3, -5, and -7 (3, 4, 45).

Rotavirus NSP1, an RNA-binding protein (21), is the only rotavirus protein implicated in evasion of innate immune response by counteracting induction of IFN to influence virus replication (4, 22); however, whether NSP1 or other rotavirus-encoded proteins modulate any other host cellular signaling pathways is not well understood. Rotaviruses have been shown to activate PI3K-mediated integrin expression and NF-κB (25, 42), but no viral protein has been reported to directly associate with these pathways. Unlike other rotavirus proteins, NSP1 is highly variable among group A rotaviruses (31), except for a conserved N-terminus cysteine-rich motif (C-X2-C-X8-C-X2-C-X3-H-X-C-X2-C-X-5-C), which is a putative zinc finger motif. Since this region is conserved, it has been postulated that it may have an important role in function of the protein. The C terminus of NSP1 has an IRF3 binding site and has been shown to be involved in IRF3 degradation (3, 4). To study the role of NSP1 in modulation of apoptosis, we utilized an NSP1 wild-type (wt) bovine rotavirus strain A5-13 and an isogenic NSP1 mutant strain, A5-16. The NSP1 of A5-16 has a 500-nucleotide deletion (nucleotides 142 to 641) in the N terminus, including the cysteine-rich zinc finger motif (Fig. 1A), followed by a nonsense codon resulting in lack of detectable functional protein (53). A5-16 is not replication defective, although it has been shown to have smaller plaque size compared to A5-13 (53). This is the first report showing that rotavirus NSP1 helps rotavirus to establish and replicate efficiently in host cells by inhibiting the cellular apoptosis through the activation of the prosurvival pathways PI3K/Akt and NF-κB during the initial stages of infection.

**MATERIALS AND METHODS**

**Reagents.** Inhibitors for PI3K (LY294002) and NF-κB (SN50) were purchased from Invivogen (San Diego, CA) and Calbiochem (San Diego, CA). Epidermal growth factor (EGF), TNF-α, and other fine chemicals and buffers were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell culture and virus infection.** The monkey kidney cell line (MA104) was cultured in minimal essential medium (MEM), VERO cell line, human intestinal epithelial (HT29), and human embryonic kidney epithelial (HEK293T) cell lines were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with U.S. certified 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA). Cells were maintained in 5% CO₂ at 37°C humidified incubator. The bovine rotavirus wild-type A5-13 and NSP1 mutant A5-16 strains were used in the study (53). In some experiments, the simian rotavirus strain SA11 and bovine rotavirus strain NCDV were used to confirm findings. For
infection, viruses were activated with acetylated trypsin (10 μg/ml) at 37°C for 30 min, diluted as required by the multiplicity of infection (MOI), and added to the cells for adsorption (45 min) at 37°C, followed by three washes with media to remove unbound virus. Infection was continued in fresh medium. Except for experiments with HEK293 cells, an MOI of 3 was used for MA104 or HT29 cells in the study. The time of virus removal was taken as 0 h postinfection (hpi) for all experiments. At different time points, cells were freeze-thawed for cell lysis. Extracted and purified (33) viral preparations were titrated by plaque assay. The end point in experiments was determined based on time required by virus to complete its replication in cells resulting in >80% cytopathic effect. The end point for MA104 cells and HT29 cells varied from 18 to 24 h, respectively. In all experiments, dimethyl sulfoxide (DMSO) was added in mock-infected controls to rule out any adverse effects of DMSO. The NF-κB or PI3K inhibitors were added, at concentrations of 5 and 10 μM, respectively, after adsorption of virus and the addition of the replication media (0 hpi).

Plaque assay. Monolayers of MA104 cells in six-well plates were infected with serial dilutions (10^2 to 10^5) of viral supernatants. After 45 min of adsorption, the inoculum was removed, and the cells were overlaid with 0.7% agar in 1× MEM with 1 μg of trypsin/ml. At 36 h to 48 hpi, a second agar overlay (0.7% agar in 1× MEM with 0.1% neutral red) was added, and the plates were incubated at 37°C until the plaques were visualized. Viral PFU were calculated as described previously (47).

Cell viability assay (MTS assay). Cell viability in the presence or absence of different inhibitors was determined by Cell titre 96 Aqueous One Solution cell proliferation assay kit (Promega, Madison, WI), which contains (MTS [3-(4, 5-dimethylthiazol-2-yl)-2,5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]) and an electron coupling reagent (phenazine ethosulfate), MTS is bioreduced to form soluble colored formazan by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Briefly, cells with ~80% confluency in 96-well plates were incubated with different inhibitors or DMSO for the indicated time periods. At the end of each incubation period the cells were treated with 20 μl of the reagent solution in 100 μl of serum-free medium for 4 h at 37°C in a humidified 5% CO₂ atmosphere. The quantity of the soluble formazan product was measured at 490 nm in a Varioskan multimode reader (Thermo Fisher). The absorbance was directly proportional to the number of living cells in culture. The percent cellular toxicity was calculated considering 100% viability for untreated control cells at similar end points.

Gel electrophoresis and immunoblot analyses. Whole-cell lysates or nuclear extracts were prepared, and immunoblotting was performed according to standard protocols (10). Rabbit polyclonal antibodies (Pabs) to Akt, GSK-3β, p-Akt (Ser473), p-GSK-3β, and PARP (Cell Signaling, Inc., Devers, MA) or mouse monoclonal antibodies to NF-κB (p65), XIAP, PI3K (BD Pharmingen, San Diego, CA), PCNA (Cell Signaling), and caspase-3 (Biomol, Plymouth Meeting, PA) were used at concentrations recommended by the manufacturer. Pabs against rotavirus nonstructural proteins were raised against peptide NSP-1 (amino acids 480 to 496) or by purification of full-length protein expressed in Escherichia coli (amino acids 480 to 496) or by purification of full-length protein expressed in an E. coli bacterial expression system (NSP-3 and NSP-5), in rabbits according to standard protocols at the Department of Virology and Parasitology, Fujita Health University, Toyoake, Japan. The antibodies were directed against the epitope sequence (amino acids 480 to 496) or by purification of full-length protein expressed in an E. coli bacterial expression system (NSP-3 and NSP-5), in rabbits according to standard protocols at the Department of Virology and Parasitology, Fujita Health University, Toyoake, Japan. The monoclonal mouse anti-rotavirus VP6 antibody (3C10) used in the work was a gift from Dr. G. A. S. Turner (Bristol-Myers Squibb). The monoclonal mouse anti-rotavirus VP6 antibody (3C10) used in the work was a gift from Dr. G. A. S. Turner (Bristol-Myers Squibb). The monoclonal mouse anti-rotavirus VP6 antibody (3C10) used in the work was a gift from Dr. G. A. S. Turner (Bristol-Myers Squibb). The monoclonal mouse anti-rotavirus VP6 antibody (3C10) used in the work was a gift from Dr. G. A. S. Turner (Bristol-Myers Squibb).

GFP expression analysis. GFP or GFP-ΔNLS expression was visualized by confocal microscopy. Cells were fixed with 4% paraformaldehyde (4% [w/v] in phosphate-buffered saline [PBS]) for 10 min at room temperature and permeabilized with 0.1% Triton X-100 for 20 min at 4°C. The samples were then incubated in blocking solution (PBS supplemented with 5% [v/v] horse serum and 5% [v/v] goat serum) for 1 h at room temperature. The cells were washed with cold PBS and incubated with monoclonal NF-κB (p65) and polyclonal RV-NSP5 antibodies at room temperature for 2 h, followed by RRX-conjugated anti-mouse and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibodies (Jackson Laboratories, Inc., West Grove, PA) for 1 h. After being washed 5 times with PBS, the slides were mounted with Vectashield-DAPI (Vector Laboratories, Burlingame, CA) and examined under a fluorescence microscope (Carl Zeiss, Gottingen, Germany). Excitation and emission detection for each fluor was performed sequentially to avoid cross talk.

RESULTS

Immunoblot analysis of A5-13- and A5-16-encoded proteins VP6, NSP3, and NSP1 after infection. To confirm the infection and replication of rotavirus strains A5-13 (wt NSP1) and A5-16 (NSP1 mutant) (Fig. 1A), in MA104 cells, immunoblot analysis was performed to analyze expression of virus encoded proteins.
After 12 h of infection at an MOI of 3, cell lysates were prepared, and the expression of VP6, NSP1, and NSP3 was analyzed by immunoblotting with specific polyclonal antibodies. The band intensities of phosphoproteins were normalized against that of total proteins. The data represent the means ± the standard deviations (SD) of three independent experiments. (B) Akt phosphorylation (ser473) level was compared by Western blotting in the 293T cell line after overexpression of full-length NSP1 (pcD-NSP1) and its truncated form (pcD-ΔNSP1) with respect to the vector-transfected control. Total Akt expression was monitored as a loading control. The NSP1 dependence of Akt activation was further verified by analyzing the pAkt level over the total Akt level in 293T cells overexpressed with full-length or truncated NSP1 protein (24 h), followed by infection with the NSP1 mutant virus A5-16 (MOI of 3) in a time-dependent manner. Consistent Akt phosphorylation (4 to 12 hpi) was found after A5-16 infection in pcD-NSP1-transfected cells, whereas in pcD-ΔNSP1 transfectants weak Akt phosphorylation was observed.

**FIG. 2.** Rotavirus-induced PI3K/Akt activation is NSP1 dependent. (A) Western blot analysis showing phosphorylation of Akt (ser473) and its downstream substrate GSK3β at 2 to 12 hpi with A5-13 and A5-16 (MOI of 3) in MA104 cells. Equal protein loading was verified by reprobing the blots to analyze the basal expression of total Akt and GSK3β. The VP6 protein level was also checked to verify the infection status of both viruses.

**ROTAVIRUS-INDUCED ACTIVATION OF THE CELLULAR PI3K/AKT PATHWAY DEPENDS ON FULL-LENGTH NSP1 EXPRESSION BY INFECTION VIRUS.** Since the PI3K/Akt pathway has been shown to be activated after many acute viral infections, we initially investigated whether NSP1 has any role in the activation of Akt (12, 25, 51). MA104 cells were infected with A5-13 and A5-16 (MOI of 3), and cell lysates were prepared at 2 to 12 hpi. The phosphorylation of Akt (ser473) was analyzed by immunoblotting. As shown in Fig. 2A, A5-13 infection resulted in rapid induction (2 ± 0.3-fold) of phospho-Akt as early as 2 hpi, with a further increase (4 ± 0.2-fold) at 4 to 12 hpi compared to the NSP1 mutant strain A5-16, which resulted in weak phosphorylation of Akt at 2 to 4 hpi. To further confirm activation of Akt, phosphorylation of its substrate glycogen synthase kinase β (GSK3β) was assessed. As expected, 2- to 4-fold less phospho-GSK3β was observed after infection with A5-16 compared to A5-13 (Fig. 2A). Similar results were obtained in intestinal epithelial cells HT29 (data not shown). To rule out effects of other rotavirus proteins or dsRNA-mediated effects, pcD-NSP1, pcD-ΔNSP1, or pcDNA6 was transfected in HEK293T cells. After 24 h, the cells were lysed and immunoblotted with phospho-Akt antibody. Phosphorylation of Akt was detected in pcD-NSP1 transfected cells only (Fig. 2B), which confirms that NSP1 alone is sufficient to activate PI3K/Akt pathway. Since weak Akt phosphorylation was observed in A5-16-infected cells (Fig. 2A), we further analyzed the effect of NSP1 expression in A5-16-infected cells. HEK293T cells transfected with either pcD-NSP1 or pcD-ΔNSP1 were infected with A5-16 (MOI of 3), and Akt phosphorylation was analyzed at different time intervals. Consistent Akt phosphorylation (4 to 12 hpi) was found after A5-16 infection in pcD-NSP1-transfected cells, whereas in pcD-ΔNSP1 transfectants weak Akt phosphorylation was observed.
tion was observed at 4 hpi (Fig. 2B, bottom panel), mimicking the effects of A5-16 infection alone.

To rule out the probability that differences in number of viral particles of A5-13 and A5-16 in cells could result in differential activation of cellular responses, MA104 cells were infected with either A5-13 or A5-16 at MOIs of 1 and 5, respectively. No significant changes were observed in Akt phosphorylation in the A5-16-infected cells at lower or higher MOIs; however, a dose-dependent increase in Akt phosphorylation was observed in A5-13-infected cells. As the internal control for viral load, the expression of VP6 was measured. VP6 expression was stronger at a higher MOI in both A5-13- and A5-16-infected cells (Fig. 2C).

Rotavirus NSP1 interacts with PI3K and modulates activation of the PI3K/Akt pathway. To confirm whether activation of PI3K/Akt is due to modulation of cellular signaling mechanism or to protein-protein interaction of NSP1 with PI3K, MA104 cells were infected with either A5-13 or A5-16 at MOIs of 1 and 5, respectively. No significant changes were observed in Akt phosphorylation in the A5-16-infected cells at lower or higher MOIs; however, a dose-dependent increase in Akt phosphorylation was observed in A5-13-infected cells. As the internal control for viral load, the expression of VP6 was measured. VP6 expression was stronger at a higher MOI in both A5-13- and A5-16-infected cells (Fig. 2C).

Rotavirus-induced Akt activation is IFN independent. Based on the reports earlier (3, 4), a comparative study was made to see the effect of NSP1 on induction of IFN or IFN-stimulated genes (ISGs). In a time course study, RNA extracted from MA104 cells infected with wt A5-13 and mutant A5-16 (MOI of 3) was subjected to real-time PCR analysis to monitor the transcripts of IFN-β and two ISGs (IRF-3 and ISG56). After infection with A5-16 strain, the IFN-β, IRF3 and ISG56 genes were induced in a time-dependent manner; however, wt A5-13 infection showed initial induction, followed by a decrease in transcripts by 8 hpi (Fig. 4A), which again confirms the earlier reports of NSP1 counteracting interferon induction.

To analyze whether the reduced IFN level in A5-13 virus-infected cells results in strong PI3K/Akt activation, Akt phosphorylation was measured in Vero cells (defective in type 1 IFN signaling) after infection (6 h and 12 h) with either wt A5-13 and mutant A5-16 strains (MOI of 10). Compared to A5-16 infection, significantly higher level of Akt phosphorylation was observed in A5-13-infected Vero cells (Fig. 4B), confirming that rotavirus NSP1-induced Akt activation is independent of IFN induction.

Functional NSP1 is required for sustained NF-κB activation. Earlier studies have reported induction of NF-κB after rotavirus infection in vitro (42). To analyze the functional role of NSP1 in modulating the activity of NF-κB, the subcellular localization of NF-κB-p65 was evaluated by immunofluorescence microscopy. MA104 cells were either mock infected or infected with A5-13, A5-16, NCDV, or SA11 strains (MOI of 3), and the presence of viral particles and p65 localization was detected by staining with anti-rotavirus NSP5 and anti-p65.
antibody (Fig. 5A) at 6 hpi. Virus infection was observed by expression of NSP5 in cells infected with any rotavirus strain (Fig. 5A, column 2). p65 was diffusely distributed throughout the cytoplasm in A5-16-infected cells, with some staining in the nucleus. Distinct nuclear staining of p65 was evident in A5-13- and SA11-infected cells, suggesting a possible role of NSP1 in NF-κB activation. However, in NCDV-infected cells, a low level of nuclear translocation compared to A5-13- and SA11-infected cells was observed (Fig. 5A, column 3). To further confirm NF-κB activation and translocation, nuclear extracts were prepared after 6 hpi, and the luciferase activity was measured. Compared to the untransfected and mock-infected control, a significant increase in luciferase activity (4- to 5-fold) was observed in A5-13-infected cells compared to the virus-infected cells in the absence of PI3K inhibitor, confirming a correlation between P13K and NF-κB signaling during virus infection. In the presence of NF-κB inhibitor SN50 (5 μM), the NF-κB activation was completely inhibited (Fig. 5C). TNF-α (10 nM), a strong activator of NF-κB, was used as a positive control for the assay.

The role of functional NSP1 in NF-κB activation was further confirmed by quantitating NF-κB promoter activity in cells expressing pcD-NSP1 or pcD-ΔNSP1. HEK293T cells were cotransfected with NF-κB-luc, pRL-TK, and pcD-NSP1 or pcDNA. After 24 h, the cells were mock infected or infected with A5-16 (MOI of 5). Cell lysates were prepared after 6 hpi, and the luciferase activity was measured. Compared to the untransfected and mock-infected control, a strong activation of NF-κB promoter activity (4- to 5-fold) was observed after transfection with pcD-NSP1 (Fig. 5D, column 2), which further increased after infection with A5-16 (Fig. 5D, column 3). The increase in luciferase activity was due to complementation by NSP1, since cells transfected with pcD-ΔNSP1 failed to activate NF-κB after A5-16 infection (Fig. 5D, column 4).

Reduced growth of NSP1 mutant virus A5-16 is due to poor induction of the PI3K/Akt and NF-κB pathways. NSP1 mutants have been reported to be replication competent but with smaller plaque sizes and slow cell-cell spread rates. Viral growth assays by conventional plaque assays were performed after infection with A5-13 and A5-16 (MOI of 3) in MA104 and HT29 cells. After 24 h the virus titers were measured. We observed 1.2- to 1.4-log and 1.8- to 2.0-log differences in the viral particles (PFU) of A5-13 and A5-16 in MA104 and HT29 cells, respectively (Fig. 6A). To confirm whether reduced number of viral particles is due to weak induction of PI3K/Akt and NF-κB, A5-13 strain was infected in MA104 and HT29 cells, followed by treatment with either P13K (LY294002; 10 μM) or NF-κB (SN50; 5 μM) inhibitors or DMSO after virus adsorp-
tion (0 hpi). Compared to the DMSO control, 1.8- to 2.0-log and 1.4- to 1.6-log reductions in A5-13 growth were observed in the presence of LY294002 and SN50, respectively (Fig. 6A). Furthermore, in the presence of the Akt and NF-κB agonists EGF (10 μM) and TNF-α (10 nM), a significant increase (2- to 3-fold) in VP6 transcripts (4 to 8 hpi) was observed compared to the cells infected with A5-16 in the absence of EGF or TNF-α (Fig. 6B), confirming a positive requirement of Akt and NF-κB for efficient virus replication. To rule out the probability of reduced viral growth due to the cytotoxic effect of inhibitors, the cytotoxicity of either LY294002 or SN50 or DMSO was analyzed by the MTS assay in both MA104 and HT29 cells in serum-free media (mimicking virus infection). As shown in Fig. 6C, at the dose used in the study, only 4.5 to 8% and 11.5 to 15% cellular toxicity was observed at 24 and 48 h after treatment with either of the inhibitors.

NSP1 supports rotavirus replication by suppressing virus-induced premature apoptosis. The regulation of antiapoptotic pathways by rotavirus NSP1 was confirmed, but whether it results in the modulation of apoptosis was analyzed by assessing the effects of A5-13 and A5-16 infection (MOI of 3) on the expression of antiapoptotic proteins XIAP (X-linked inhibitor of apoptosis) or the cleavage of PARP and caspase-3 by immunoblotting in MA104 cells. There was a 3- to 4-fold induction in expression of XIAP at 4 to 12 hpi with A5-13 strain compared to the transient (0.6- to 0.3-fold) induction with A5-16 (Fig. 7A). In contrast to low XIAP expression, the A5-16 strain showed enhanced cleavage of caspase-3 as early as 8 hpi with a 6- to 8-fold increase by 12 hpi. The inactivated 85-kDa PARP fragment was also 2.5- to 4-fold higher in A5-16-infected cells compared to A5-13-infected cells (Fig. 7B). In A5-13-infected cells, caspase-3 cleavage was observed during later stages of infection (12 to 18 hpi). This was further confirmed by quantitating the protease activity of caspase-3 by using a fluorometric assay. There was a significant increase in caspase-3 activity (2- to 3-fold) at an early stage of infection (4 to 8 hpi) in mutant strain A5-16-infected cells compared to the A5-13 infection (Fig. 7C). At 12 to 18 hpi, the caspase-3 ac-
activity in A5-13-infected cells increased significantly relative to mock-infected controls. At 18 hpi, the caspase activity was higher in A5-16-infected cells during later stages. The caspase-3 inhibitor DEVD-fmk was used as an internal control to confirm assay validity. The caspase-3 enzyme activity was also measured in cells infected with A5-13 in the presence of PI3K/Akt and NF-κB inhibitors. The data represent the means of three experiments (n = 3, P < 0.05). (B) Enhanced viral gene expression in the presence Akt and NF-κB agonists (EGF [10 μM] and TNF-α [10 nM]). VP6 transcripts were quantitated by qRT-PCR after A5-16 infection in either Akt or NF-κB agonist-treated MA104 cells or untreated MA104 cells. The data represent means ± the SD (n = 3, P < 0.01). (C) The percentage of cytotoxicity was measured by MTS assay in MA104 and HT29 cells in serum-free conditions, after treatment with LY294002 (10 μM), SN50 (5 μM), or DMSO (control) in a time-dependent manner. In both cell lines, 4.5 to 7% and 11.5 to 17% cytotoxicities were observed with either inhibitor at 24 and 48 h of treatment, respectively.

DISCUSSION

Viruses in general depend on host cellular machinery, and thus the interactions of viral proteins with the cellular proteins are well documented. The activation of signals such as PI3K/Akt and NF-κB has been reported during pathogen infections (2, 6, 17, 39, 42, 46). After rotavirus infection, the regulation of integrin expression in intestinal cells has been shown to be PI3K dependent (25). PI3Ks, a family of heterodimeric enzymes are activated by binding to Src homology-2 or -3 (SH2 or SH3) domains of the proteins to the p85 or p110 subunits (13). Activated PI3K can further activate downstream protein or lipid kinases, including Akt. Activated Akt plays a central role in modulating diverse downstream signaling pathways associated with differentiation, proliferation, and the prevention of apoptosis (13). In the case of viruses causing acute infections, such as dengue virus, influenza virus, coxsackievirus, vaccinia virus, and cowpox virus, etc., PI3K/Akt signaling has been reported to assist viral replication by inhibiting apoptosis (2, 16, 17, 20, 39, 48). Influenza A virus infection has been shown to activate the PI3K/Akt pathway by direct interaction of the SH3 binding motif of NS1 protein with the p85 subunit of PI3K.
FIG. 7. Rotavirus NSP1 inhibits virus-induced premature apoptosis. MA104 cells were infected with either wt A5-13 or mutant A5-16 (MOI of 3). Cell lysates were prepared at increasing time intervals, and Western blot analysis was performed to detect the expression of antiapoptotic or apoptotic proteins: XIAP (A) and caspase-3 and PARP (B). β-Actin was used as an endogenous control for equal protein loading. (C) Caspase-3 activity was measured in MA104 cells infected with wt A5-13 or mutant A5-16 strains, using the fluorogenic substrate DEVD-AFC, revealed higher caspase-3 activities during the initial stages of infection in A5-16-infected cells compared to A5-13-infected cells. Caspase-3 inhibitor DEVD-fmk was used as an internal control. (D) Caspase-3 enzyme activity as measured in MA104 cells infected with A5-13 (MOI of 3) in the presence of PI3K inhibitor (LY294002, 10 μM), NF-κB inhibitor SN50 (5 μM), or DMSO as a control. In the presence of PI3K or NF-κB inhibitors, increased caspase-3 activity was observed as early as 8 hpi. (E) DNA fragmentation was measured by TUNEL assay by flow cytometry analysis. MA104 or HT29 cells were infected with either wt A5-13 or mutant A5-16 (MOI of 3) or mock infected for 8, 12, and 18 h or 8, 16, and 24 h, respectively, and incubated with terminal deoxynucleotidyltransferase and FITC-conjugated anti-BrdU monoclonal antibodies according to standard protocols (BD Pharmingen). In A5-16-infected MA104 or HT29 cells, increased TUNEL positivity was observed during earlier stages of infection compared to A5-13-infected or mock-infected controls. The data shown represent means ± the SD (n = 4).

In the present study, we observed the role of rotavirus NSP1 in sustained PI3K/Akt activation in MA104 and HT29 cells since mutant virus could only transiently activate PI3K (Fig. 2A). Transient activation of PI3K/Akt by A5-16 was not probably due to the truncated 50-amino-acid NSP1 since pcD-ΔNSP1 failed to induce the phosphorylation of Akt (Fig. 2B). Since increasing concentrations of A5-16 (MOI of 5) could not further activate Akt (Fig. 2C), the role of other viral proteins can be ruled out, and the transient activation of Akt is probably due to activation of the innate immune responses during pathogen infection. The phosphorylation of Akt in pcD-NSP1-transfected HEK293T cells indicates that NSP1-modulated PI3K/Akt activation occurs irrespective of the cell type (Fig. 2B). pcD-NSP1 was functional since IRF3 degradation was observed after A5-16 infection of pcD-NSP1-transfected cells (see Fig. S6 in the supplemental material); however, due to poor expression of NSP1, the dose-dependent effect of pcD-NSP1 transfection on Akt activation could not be assessed. Difference in Akt activation by A5-13 or A5-16 was also not due to variable viral dsRNA content, since no significant differences were observed in transcript levels of NSP4 or VP6 during the early hours of the infection (2 to 6 hpi) with either strain (see Fig. S1A in the supplemental material). It is well documented that PI3K, the upstream activator of Akt, is commonly stimulated upon activation of membrane receptors that either couple to heterotrimeric GTP proteins or have tyrosine kinase activity (28, 40). However, Psoralene-UV-irradiated A5-13 (which is replication deficient and noninfectious but is a structurally and immunologically competent virus) (42), could not activate the PI3K/Akt pathway efficiently (see Fig. S1B in the supplemental material), suggesting that activation of the PI3K/Akt pathway during rotavirus infection is more likely induced directly or indirectly by viral proteins during replication.

Coimmunoprecipitation of NSP1 with the p85 subunit of PI3K after A5-13 infection or pcD-NSP1 transfection suggested that activation of PI3K could be due to an interaction of NSP1 with the endogenous PI3K (Fig. 3). Sequence analysis of the NSP1 gene of prototype rotavirus strains revealed the presence of at least one putative SH2 binding motif (YXXXM), which is a putative PI3K binding site (49). An association of cellular PI3K with NSP1 was further confirmed by colocalization of PI3K in cells expressing eGFP-NSP1 but not with eGFP-ΔNSP1 (see Fig. S2 in the supplemental material). However, whether this interaction of NSP1 with PI3K is due to direct binding or is a part of a multiprotein complex remains to be elucidated.

Confirming previous observations, a time-dependent induction of IFN-β and other ISG transcripts following the NSP1 mutant A5-16 was observed (Fig. 4A). In A5-13-infected cells, IRF3 and IFN-β were upregulated as early as 2 hpi, but 2-fold inhibition was observed by 4 hpi, a finding which correlates with previous reports of the role of NSP1 in the modulation of IRF3 and IFN (3, 4) (Fig. 4A). This was further confirmed in the present study since ISG56, an IRF3- and IFN-regulated gene, was significantly upregulated at 2 hpi (4-fold) and 4 hpi (10-fold), followed by downregulation at 8 hpi in A5-13-infected cells (Fig. 4A). Inhibition of PI3K has been shown previously to enhance TRIF-dependent IFN-β induction (1), and rotavirus NSP1 is an IFN antagonist; thus, one could
speculate the inverse relationship between IFN and PI3K/Akt activation. However, induction of Akt phosphorylation during A5-13 but not with A5-16 infection in the Vero cell line was observed (Fig. 4B), suggesting that rotavirus-induced PI3K/Akt activation is an IFN-independent phenomenon.

Degradation of IRF3 was observed by immunoblotting, following infection of MA104 cells with A5-13 but not with A5-16 (data not shown). In addition, A5-16 infection in pcd-NSP1-expressing cells resulted in the degradation of IRF3 (see Fig. S6 in the supplemental material), as well as the activation of Akt (Fig. 2B). Thus, although the results confirm the role of NSP1 in the activation of Akt and the degradation of IRF3, it will be interesting to determine whether direct interactions between NSP1 and PI3K or between NSP1 and IRF3 or whether the degradation of IRF3 individually or cooperatively triggers the activation of PI3K after rotavirus infection.

Activation of NF-κB is a strategy adopted by many viruses, namely, HIV, herpesviruses, and encephalomyocarditis virus (44), to block early premature apoptosis and prolong survival of the host cell to gain time for replication and the increase of viral progeny. Activation of NF-κB in HT29 cells after rotavirus infection or only the nonreproducibly double-layered particles has been previously reported (37, 42). In the present study, we observed sustained activation and nuclear translocation of NF-κB after A5-13 infection, which is consistent with previous reports on NF-κB activation after rotavirus infection (15, 37, 42, 43). In spite of a previous report of the nuclear translocation of NF-κB in A5-16 (24), compared to A5-13, we observed significantly (>4-fold) lower nuclear translocation of p65 NF-κB (Fig. 5A and B) or NF-κB promoter activation (Fig. 5C) in A5-16-infected cells. Nuclear translocation of NF-κB was also observed after infection with rotavirus strains SA11 and NCDV, although the levels of translocation varied among the strains, since at 6 hpi the NCDV-infected cells showed significantly less nuclear translocation of NF-κB compared to A5-13 and SA11 (Fig. 5A and B), whereas at early time points (1 to 2 hpi), comparable levels of NF-κB activation were observed in NCDV cells (Fig. 5B).

Lower levels of nuclear translocation of NF-κB in NCDV-infected cells are probably due to proteasome-dependent degradation of βTrCP by NCDV NSP1 (24) since, similar to our observations with A5-13 or SA11, we observed significant Akt activation in NCDV-infected cells (see Fig. S5 in the supplemental material). In another study, the activation of NF-κB was reported during early time of infection; however, after 8 hpi, no nuclear translocation of NF-κB was observed, even after TNF-α treatment (30). This inhibition is probably due to a negative-feedback mechanism to regulate the expression of antiviral responses such as the induction of interleukin-8. In spite of differences observed in the time or levels of NF-κB activation among rotavirus strains, most strains show NF-κB activation during the early stages of infection (24, 30, 37, 42).

It is possible that NF-κB is initially activated as part of an antiviral innate immune response upon virus infection; however, NSP1 may have a role in stabilizing the activated NF-κB for delaying the cellular apoptosis to establish virus infection. The observed NSP1 dependence of NF-κB activation was further substantiated by Western blot analysis showing IκBα degradation in MA104 cells infected with A5-13 rather than A5-16 (see Fig. S3A in the supplemental material) during the early hours (4 to 8 h) of infection. No IκBα degradation was observed in the presence of MG132 (see Fig. S3B in the supplemental material), suggesting that the degradation of IκBα in the presence of NSP1 is proteasome dependent. Sustained activation of NF-κB is dependent on functional NSP1 since the activation of NF-κB promoter activity was restored when A5-16 was used to infect cells expressing pCD-NSP1 but not in cells expressing pCD-ΔNSP1 (Fig. 5D). Virus infection has also been shown to activate IFN transcriptional elements such as ISGF3 and STAT1 (42), resulting in PKR activation, leading to the activation of NF-κB (36). However, during rotavirus infection, the kinetics of the activation of PKR or STAT1 lagged behind the appearance of nuclear NF-κB (<2 hpi), suggesting the possibility that the initial NF-κB activation is independent of IFN signaling (42).

Activated AKT phosphorylates a large number of substrates, such as glycogen synthase kinase 3β (GSK3β) and IκB kinases. This can lead to inactivation of the proapoptotic GSK3β and activation of antiapoptotic pathways such as IκB kinase-mediated activation of NF-κB. To confirm whether poor activation of the PI3K/Akt pathway by A5-16 results in weak activation of NF-κB, its activation was measured in the presence or absence of PI3K inhibitor in A5-13-infected cells. As shown in Fig. 5C, LY294002 treatment resulted in a 2.5- to 3.0-fold reduction in NF-κB promoter activity at 6 hpi compared to untreated cells. Based on these observations, we hypothesize that an initial induction of NF-κB during rotavirus infection is probably due to the VP4-TRAF2 interactions (37), but sustained activation is probably PI3K/Akt dependent (15, 41, and 54).

NS1 and NS2 deletion mutant RSV strains and NS1 mutant influenza virus have been shown to exhibit attenuated growth and small plaque morphology in vitro (6, 14). Similarly, NSP1 mutant strains of rotaviruses have also been reported to have smaller plaque sizes; this has been attributed to the suppression of cell-to-cell spread (3, 53). Consistent with NS mutants of influenza virus or RSV, we also observed reduced growth of A5-16 strain compared to A5-13 (Fig. 6A). To confirm whether weak induction of PI3K/Akt or NF-κB by NSP1 mutant was one of the probable reasons for slow growth, the A5-13 strain was used to infect in MA104 and HT29 cells and grown in the presence of either PI3K/Akt or NF-κB inhibitors. However, no significant changes in transcripts of IFN or ISGs was observed after rotavirus infection (4 to 8 hpi) in the presence of LY294002 (see Fig. S4 in the supplemental material), confirming that the activation of PI3K/Akt or IFN is independently induced during virus infection. Moreover, pretreatment of cells with activators of PI3K and NF-κB pathways, followed by A5-16 infection, resulted in a significant increase in viral gene transcription, confirming that activation of PI3K/Akt and NF-κB is required for the efficient replication of rotavirus (Fig. 6B).
Since inhibitors of PI3K/Akt and NF-κB result in a substantial decrease in virus multiplication, it is likely that increased apoptosis in A5-16-infected cells is a direct consequence of the reduced prosurvival function of Akt and NF-κB. Poor induction of the NF-κB-regulated antiapoptotic gene XIAP after A5-16 infection reflects the inability of NSP1 mutant to induce antiapoptotic machinery (Fig. 7A). Thus, to correlate the reduced viral growth with cellular apoptosis, caspase-3 and PARP cleavage was measured. The sequential activation of caspasases plays a central role in the execution phase of apoptosis. Caspasases exist as inactive proenzymes that undergo proteolytic processing at conserved aspartic acid residues to produce two subunits, large and small. Significantly high levels of caspase-3 and PARP (85 kDa) cleavage or caspase-3 protease activity were observed as early as 8 hpi in cells infected with the A5-16 strain compared to infection with A5-13 (Fig. 7B and C). The role of the PI3K/Akt pathway in delaying apoptosis in wt A5-13 was confirmed; as in the presence of PI3K/AKT or NF-κB inhibitors, 2.5- to 3-fold-higher caspase-3 activities were observed at 8 hpi after A5-13 infection (Fig. 7D). DNA fragmentation is considered a hallmark of apoptosis since during necrosis no DNA fragmentation is observed. DNA fragmentation measured by the TUNEL assay showed higher numbers of apoptotic cells in A5-16-infected MA104 and HT29 cells compared to infection with A5-13 during the initial stages of infection (Fig. 7E). These results are consistent with the reports showing a role for NS proteins in suppressing premature apoptosis during infection with RSV, poliovirus, influenza A virus, etc. (2, 6, 14, 16, 17, 39, 54). Delayed apoptosis could encourage virus growth many ways. The loss of structural integrity during apoptosis may affect viral growth, since cellular genes, cytoskeletal components, etc., are required for optimal replication (34). In addition, if the infected cell dies due to apoptosis prior to complete formation of packaged infectious virions, the released immature virus particles will be largely noninfective, resulting in aborted virus growth (6).

Thus, based on our observations and published reports, it is apparent that NSP1 may not have a direct role in replication, but it indirectly supports viral growth by counteracting the host immune responses in more than one way (Fig. 8). The role of NSP1-mediated degradation of IRF3, IRF5, and IRF7, the inhibition of IκB degradation by inducing the proteasome-dependent degradation of β-TrCP, and the inhibition of IFN activation, resulting in the inhibition of induction of antiviral response, have all been established (3, 4, 24). IFNs can act as both proapoptotic or antiapoptotic cytokine, depending on the signaling environment, although in general IFN-induced apoptosis usually occurs after 24 h (9). The relationship between IFN and apoptosis in rotavirus-infected cells is still unknown. Based on the findings of the present study we can propose that, in addition to degrading IRF3, NSP1 is also involved in the activation of PI3K/Akt. Activated Akt can inhibit proapoptotic pathways by the activation of NF-κB (41), as well as by regulating other client proteins such as GSK3β, caspases, etc., resulting in the suppression of premature apoptosis of infected cells for better growth of intracellular viruses (16) (Fig. 8). Thus, both of the NSP1-regulated pathways exist in parallel and can function simultaneously, independently, or in a partially overlapping and cooperative manner to positively support virus infection. Additional rotavirus-regulated cellular pathways need to be explored in order to more clearly understand viral pathogenesis.

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