Rotavirus-Encoded Nonstructural Protein 1 Modulates Cellular Apoptotic Machinery by Targeting Tumor Suppressor Protein p53

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p53, a member of the innate immune system, is triggered under stress to induce cell growth arrest and apoptosis. Thus, p53 is an important target for viruses, as efficient infection depends on modulation of the host apoptotic machinery. This study focuses on how rotavirus manipulates intricate p53 signaling for their advantage. Analysis of p53 expression revealed degradation of p53 during initial stages of rotavirus infection. However, in nonstructural protein-1 (NSP1) mutant strain A5-16, p53 degradation was not observed, suggesting a role of NSP1 in this process. This function of NSP1 was independent of its interferon or phosphatidylinositol 3-kinase (PI3K)/AKT modulation activity since p53 degradation was observed in Vero cells as well as in the presence of PI3K inhibitor. p53 transcript levels remained the same in SA11-infected cells (at 2 to 14 h postinfection), but p53 protein was stabilized only in the presence of MG132, suggesting a posttranslational process. NSP1 interacted with the DNA binding domain of p53, resulting in ubiquitination and proteasomal degradation of p53. Degradation of p53 during initial stages of infection inhibited apoptosis, as the proapoptotic genes PUMA and Bax were downregulated. During late viral infection, when progeny dissemination is the main objective, the NSP1-p53 interaction was diminished, resulting in restoration of the p53 level, with initiation of proapoptotic signaling ensuing. Overall results highlight the multiple strategies evolved by NSP1 to combat the host immune response.

The p53 protein was first identified in simian virus 40-transformed cells as a T antigen-associated cellular protein and characterized as the first vertebrate oncogene on the basis of its sequence similarity to the cancer-causing gene of a chicken retrovirus (1–3). Although from the initial days of discovery p53 was related to cancer and coined a tumor suppressor gene, recent studies have delineated its role in other aspects of life, such as the development, life expectancy, and overall fitness of an organism (4). p53 is a stress-responsive transcription factor that controls genes involved in the cell cycle, apoptosis, DNA repair, and angiogenesis (5). Since p53 is an important regulator of cellular processes, the level of p53 is controlled by several complex mechanisms and feedback loops. Under unstressed condition, p53 remains in the hypophosphorylated form, which gets degraded by the MDM2 protein; however, in response to stress, phosphorylation of p53 occurs, resulting in inactivation of ubiquitin (Ub)-mediated degradation, which leads to rapid p53 accumulation in the nucleus, where it functions as a transcription factor.

For evading host antiviral machinery and creating a beneficial environment for viral replication and dissemination, viruses have evolved measures to target key cellular genes, such as interferon-regulatory factor3 (IRF3) (6), p53 (7), the alpha subunit of eukaryotic initiation factor 2 (eIF2alpha) (8), and NF-kB (9). By targeting p53, viruses can control an important innate immune response, namely, apoptosis, for their own advantage. There are reports of virus-induced downregulation of p53 by degradation (10–12), inactivation of p53 transactivation (13, 14), as well as stabilization of p53 (15–17), depending on the virus type or stage of viral replication.

Rotavirus, a Reoviridae family member, is the most important etiologic agent of severe infantile (age, <5 years) nonbacterial diarrhea in humans worldwide (18). It is a nonenveloped icosahedral structured virus having 11 segments of double-stranded RNA which remain concealed by 6 structural proteins (VP1 to VP4, VP6, VP7). In addition, the virus produces 6 nonstructural proteins (NSP1 to NSP6) after infection. These mainly control the host machinery and play a vital role in establishing infection by carrying out diverse functions. Among them, NSP1, an RNA binding protein (18), has been shown to activate the phosphatidylinositol 3-kinase (PI3K)/AKT-mediated antiapoptotic pathway (19) as well as to inhibit innate immune responses by degradation of IRFs and RIG-I (20, 21), resulting in efficient virus infection and replication. In addition to its ability to bind the p85 subunit of PI3K for activation of AKT, much circumstantial evidence putatively suggests that NSP1 also has ubiquitin ligase properties (22, 23).

Ubiquitination is the main process for intracellular protein degradation in eukaryotes (24). Ubiquitin ligases recognize and bind to target proteins and label them with ubiquitin, which is recognized by the proteasomal machinery. In spite of reports on the significance of p53 during virus infection, especially in oncoviruses, not much is known about its role in the self-limitation of enteric viruses, such as rotavirus. In our study, we show that during initial stages of rotavirus infection, the NSP1 protein targets p53 for ubiquitination and degradation, resulting in an antiapoptotic atmosphere in the infected cell, but during late infection, the interaction between p53 and NSP1 is diminished, re-
resulting in activation of the p53-regulated protein Bax and PUMA, leading to induction of apoptosis.

**MATERIALS AND METHODS**

**Viruses, cells, and viral infection.** Monkey kidney (MA104) cells were cultured in minimal essential medium (MEM). Cells of the human embryonic kidney epithelial cell line (HEK293T [293T]) and Vero cell line were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1/100 PSF (penicillin, streptomycin, and amphotericin B [Fungizone]) at 37°C in a humidified incubator with 5% CO₂. Rotavirus strains SA11 and A513 and NSP1 mutant strain A5-16 (gifts from N. Kobayashi, Japan) were used in this study. For infection, viruses were activated with acetylated trypsin (10 g/ml) at 37°C for 30 min and added to the cells at a multiplicity of infection (MOI) of 3 for 45 min at 37°C. Unbound virus was removed by washing with medium, and infection was continued in fresh MEM supplemented with acetylated trypsin and antibiotic. The time of virus removal was taken as 0 h postinfection (hpi) for all experiments. Extracted and purified viral preparations were titrated by plaque assay (18).

**Plasmid construction.** The p53 vector set containing pCMV-p53 and dominant negative pCMV-p53-mt135 (DNp53; catalog no. 631922) was purchased from Clontech. NSP1 p53 and different truncated mutants of NSP1 p53 were cloned in pcDNA6B and ubiquitin, and the domains of p53 were cloned in the pFLAG-CMV vector using specific primers.

**Antibodies, reagents, and inhibitor.** Rabbit polyclonal antibody against NSP1 was raised against a peptide fragment of NSP1 according to standard protocols at the Department of Virology and Parasitology, Fujita Health University School of Medicine, Aichi, Japan. Antibodies against the His probe (sc-803) and Bax (sc-493) were from Santa Cruz Biotechnology (CA). Antibodies against caspase-9 (catalog no. 9501 and 9502), caspase-3 (catalog no. 9662 and 9664), poly(ADP-ribose) polymerase (PARP (catalog no. 9541 and 9542), Cox4 (catalog no. 4844), GAPDH (glyceraldehyde-3-phosphate dehydrogenase; catalog no. 2118), PUMA (catalog no. 4976), and -actin (catalog no. 4967) were from Cell Signaling Technology. Antibody against p53 (catalog no. 554165) was purchased from BD Biosciences. Antibody against the FLAG epitope (catalog no. SAB4200071) was from Sigma (St. Louis, MO). All antibodies were used at the manufacturers’ recommended dilution. PI3K inhibitor LY294002 (catalog no. 9901) was purchased from Cell Signaling Technology. MDM2 inhibitor Nutlin-3 (catalog no. N6287) was purchased from Sigma.

**Mitochondrion isolation.** Mitochondria were isolated from infected MA104 cells by the differential centrifugation method. Cells were washed with cold phosphate-buffered saline (PBS), scraped, and resuspended in 1

**FIG 1** p53 level undergoes posttranscriptional depletion during SA11 infection. (A and B) MA104 cells (A) or Vero cells (B) were infected with SA11 at an MOI of 3 for 2 to 14 hpi, followed by immunoblotting with anti-p53, anti-NSP1, and anti-GAPDH antibody. The relative fold change in protein levels of p53 during SA11 infection in both MA104 cells (A) and Vero cells (B) was normalized relative to the level of the internal control, GAPDH. Results shown here are representative of triplicate immunoblotting experiments. (C) The level of p53 remains stable in mock-infected MA104 cells. Mock-infected MA104 cells were harvested at different time points, followed by immunoblotting with p53-specific and GAPDH-specific antibody. (D) Relative fold change in transcript levels of nsp4, bax, p53, and puma during SA11 infection compared with the level of the uninfected control normalized with respect to that of the internal control, the GAPDH gene. MA104 cells were infected with SA11 at an MOI of 3 for the indicated time points. RNA was isolated, and the nsp4, p53, bax, and puma mRNA levels were analyzed by quantitative reverse transcription-PCR. Fold changes were obtained by normalizing the relative gene expression to that of the GAPDH gene using the formula 2ΔΔCT (ΔΔCT = ΔCT for the sample minus ΔCT for the untreated control).
to 2% (wt/vol) Triton X-100, 0.01 to 0.03% (wt/vol) NP-40, 0.4 to 0.6% (wt/vol) CHAPS [3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate] in ice for 30 min for cell disruption, followed by centrifugation at 1,000 \( \times g \) for 10 min. Supernatants were collected and centrifuged at 7,000 \( \times g \) for 10 min to pellet the mitochondria, and the supernatant was saved as the cytoplasm. The pellet was washed with buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 120 mM dithiothreitol (DTT), 2% ampholytes (pH 3 to 10), and 40 mM Tris-HCl and further incubated in ice for 30 min.

**Immunoblot analysis.** Whole-cell lysates (extracted with Totex buffer [20 mM HEPES at pH 7.9, 0.35 M NaCl, 20% glycerol, 1% NP-40, 1 mM MgCl\(_2\), 0.5 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 0.3 mM Na\(_3\)VO\(_4\)] containing a mixture of protease and phosphatase inhibitors [Sigma, St. Louis, MO]), cytoplasmic or mitochondrial extracts, in vitro-transcribed and -translated (IVT) products, or immunoprecipitated products were prepared. Samples were incubated in protein sample buffer (final concentrations, 50 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.01% bromphenol blue) for 30 min at 4°C or, alternatively, boiled for 5 min before SDS-PAGE at room temperature, followed by immunoblotting with specific antibodies according to the manufacturer’s recommended dilutions, as described previously (25). For anti-NSP1 antibody, a 1:3,000 dilution was used. Primary antibodies were identified with horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce, Rockford, IL) and chemiluminescent substrate (Millipore, Billerica, MA). Necessary, to confirm protein loading, blots were reprobed with β-actin, GAPDH, or Cox4. The immunoblots shown are representative of three independent experiments.

**Coimmunoprecipitation.** Cell lysates from infected MA104 cells or transfected 293T cells were clarified, followed by incubation with specific antibodies overnight at 4°C and with protein A-Sepharose beads for 4 h. Beads were washed 5 times with 1 ml wash buffer (200 mM Tris, pH 8.0, 100 mM NaCl, 0.5% NP-40), and bound proteins were eluted by boiling for 5 min with SDS sample buffer before separation on 12% SDS-polyacrylamide gels, followed by immunoblotting with specific antibodies.

**Quantitative real-time PCR.** Total RNA was isolated using the TRIzol reagent (Invitrogen, Grand Island, NY) according to the manufacturer’s instructions. CDNA was prepared from 1 to 2 μg of RNA using SuperScript II reverse transcriptase (Invitrogen) with random hexamer primers. Real-time PCRs (50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 10 min) were performed in triplicate using SYBR green (Applied Biosystems, Foster City, CA) with primers specific for the NER (forward [F] primer 5'-TACCACATCCTGATACACTG-3' and reverse primer 5'-CTACAAACGCAGCTGACAC-3') genes. The relative levels of gene expression were normalized to the level for the GAPDH gene using the formula

\[
\Delta C_T = C_T(E) - C_T(GAPDH)\]

\[
\Delta C_{T,F} = \Delta C_T - \Delta C_T(GAPDH)\]

The relative levels of gene expression were normalized to the level for the GAPDH gene using the formula

\[
\text{Relative expression} = 2^{-\Delta C_{T,F}}\]

where \( \Delta C_{T,F} \) is the change in the threshold cycle value [ΔC\(_T\)] for the sample minus ΔC\(_T\) for the untreated control.

**In vitro coupled transcription and translation and purification.** Full-length p53, NSP1, and mutants of p53 cloned under the T7 promoter (pCDNA) were subjected to in vitro coupled transcription/translation using a TNT quick coupled transcription/translation system (Promega Corporation, Madison, WI), according to the manufacturer’s specifications. In the presence of Transcend biotinylated-lysyl tRNA, 2 μg plasmid was added to TNT Quick Master mix for 90 min at 30°C and the products were...
separated by SDS-PAGE and immunoblotted using Pierce high-sensitivity streptavidin-HRP (Thermo Scientific, Rockford, IL). Recombinant proteins were purified on Ni²⁺/H₁₁₀₀₁⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~-nitrilotriacetic acid (NTA) magnetic agarose beads under native conditions. Purity was confirmed by immunoblotting with specific antibodies (data not shown).

In vitro interaction. One of the suspected purified interacting partners was subjected to enterokinase (EK) treatment (substrate ratio, 1:42 at 37°C in 50 mM Tris, pH 7.6; the reaction was stopped by adding 1 mM phenylmethylsulfonyl fluoride and heating at 95°C for 10 min, and then the reaction mixture was passed through the Ni²⁺/H₁₁₀₀₁⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~-NTA column) to remove the His tag, and the other partner (5 g) had previously been immobilized on Ni²⁺/H₁₁₀₀₁⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~-NTA by overnight incubation in HEPES-buffered saline (HBS) at 4°C. After extensive washing in PBS–0.3% Tween to remove unbound protein, immobilized protein was incubated with enterokinase-treated protein (10 g) for 4 h in HBS. Beads were washed extensively with HBS to remove nonspecifically bound proteins. Remaining proteins were separated by 4X sample buffer and then analyzed by SDS-PAGE and immunoblotted for enterokinase-treated protein.

Assay for caspase-9 and caspase-3 activation. After the different treatments, cell lysates were isolated and caspase-9 and caspase-3 cleavage activity was measured using an ApoAlert caspase fluorescent assay kit, according to the manufacturer’s protocol (Clontech, Mountain View, CA), which uses hydrolysis of the synthetic substrates Leu-Glu-His-Asp (LEHD) conjugated to 7-amino-4-methyl coumarin (LEHD-AMC) and Asp-Glu-Val-Asp (DEVD) conjugated to 7-amino-4-trifluoromethyl coumarin (DEVD-AFC), respectively.

Statistical analysis. Data are expressed as means ± standard deviations (SDs) of at least three independent experiments (n ≥ 3). In all tests, a P value of 0.05 was considered statistically significant.

RESULTS

Reduced p53 levels during SA11 infection are a posttranscriptional phenomenon. The effect of rotavirus infection on the p53 level was initially analyzed in vitro. MA104 cells were infected with SA11 (MOI, 3) at increasing time points (0 to 14 hpi), followed by immunoblot analysis of the cell lysates with p53-specific antibody. Unlike mock-infected cells (Fig. 1C), a decrease in the levels of p53 during early infection (4 to 8 hpi) followed by an increase in p53 amount at later time points (10 to 14 hpi) (Fig. 1A) was observed. To overrule the effect of rotavirus (SA11)-mediated downregulation of interferon (IFN), which in turn can modulate p53 expression (26), p53 levels were also assessed in an IFN pathway-defective Vero cell line (27). Consistent with previous results in MA104 cells, depletion of the p53 level was also observed in SA11-infected Vero cells, suggesting that the reduction in p53 level is IFN independent (Fig. 1B). To assess whether rotavirus-mediated regulation of p53 is transcriptional or posttranscriptional, p53 mRNA was quantitated by real-time PCR in SA11-infected MA104 cells (0 to 14 hpi). As shown in Fig. 1D, p53 mRNA was induced during early infection (4 to 8 hpi), and the level remained almost the same until 14 hpi, suggesting that depletion of the p53 level is a posttranscriptional phenomenon. This was further confirmed by measuring downstream p53-regulated genes, such as bax and puma. Consistent with the p53 protein level (Fig. 1A), the bax and puma genes were downregulated (Fig. 1D) during early infection.

NSP1 induces PI3K activation-independent but proteosome-dependent p53 degradation. Rotavirus NSP1 has been...
shown to degrade antiviral proteins IRF3 and RIG-I (20, 21). To assess whether the decrease in p53 level during rotavirus infection is mediated by NSP1, the levels of p53 were analyzed in MA104 cells infected with NSP1 mutant strain A5-16 (28). As shown in Fig. 2A, no p53 degradation was observed at 2 to 14 hpi following A5-16 infection, suggesting a role of NSP1 in modulation of the p53 level. For further confirmation, 293T cells were either transfected with pCMV-p53 alone or cotransfected with pCMV-p53 or cotransfected with pCMV-p53 plus pFLAG-CMV-Ub, and whole-cell lysates were immunoblotted with anti-p53 antibody, followed by immunoblotting with anti-FLAG antibody. Whole-cell lysates were immunoblotted with anti-p53 and anti-NSP1 antibody.

Our group previously reported the role of NSP1 in activation of the PI3K/AKT pathway (19). AKT has been shown to modulate functions of MDM2, a cellular ubiquitin ligase which can induce p53 degradation (29,30). To understand whether NSP1-mediated AKT phosphorylation results in p53 degradation, MA104 cells were infected with SA11 or mock infected in the presence or absence of the PI3K inhibitor LY294002 (10 μM). As shown in Fig. 3A, the p53 level was restored in the presence of MG132, whereas in the absence of MG132, p53 was degraded in NSP1-expressing cells, suggesting that p53 is degraded by a proteasome-dependent mechanism (Fig. 2C). Our group previously reported the role of NSP1 in activation of the PI3K/AKT pathway (19). AKT has been shown to modulate functions of MDM2, a cellular ubiquitin ligase which can induce p53 degradation (29,30). To understand whether NSP1-mediated AKT phosphorylation results in p53 degradation, MA104 cells were infected with SA11 or mock infected in the presence or absence of the PI3K inhibitor LY294002 (10 μM). As shown in Fig. 3A, LY294002 could not restore the levels of p53 in SA11-infected cells. Furthermore, in p53-overexpressing 293T cells following transfection with pcDNSP1, p53 degradation was observed in both the presence and absence of PI3K inhibitor (LY294002) (Fig. 3C). Similarly, in the presence of MDM2 inhibitor (Nutlin-3),
NSP1-mediated p53 degradation was not completely inhibited (Fig. 3B and D). Overall, the results suggested that the NSP1-mediated degradation of p53 is independent of its PI3K-activating function.

NSP1 interacts with p53 and modulates its ubiquitylation. To delineate the mechanism behind NSP1-mediated p53 degradation, the possibility of an interaction between the two proteins was analyzed. 293T cells were either cotransfected with pcDNSP1 and each of the p53 domains or transfected individually. After 36 h, immunoprecipitation was done with either anti-p53 antibody or anti-His antibody (for NSP1), as described in Materials and Methods, followed by immunoblotting with reciprocal antibody. Whole-cell lysates were immunoblotted with anti-NSP1 and anti-FLAG antibody. (B and C) The region from amino acids 100 to 300 interacts with NSP1 in vitro. Different IVT products of p53 mutants or IVT NSP1 were immobilized with Ni⁺², and enterokinase-treated reciprocal IVT proteins were incubated with them, followed by immunoblot analysis of the proteins interacting with the immobilized proteins.

p53 interacts with NSP1 by its DNA binding domain. According to functionality, p53 has been divided into three domains: the N-terminal transactivation domain (amino acids 1 to 80), the central DNA binding core domain (amino acids 100 to 300), and the C-terminal multifunctional regulatory domain (amino acids 300 to 393) (31,32). To identify the region which interacts with NSP1, all three domains of p53 were cloned individually in the pFLAG-CMV vector. The expression of the constructs was tested by immunoblotting with anti-FLAG antibody (Fig. 5A). To determine the interacting domains of p53, pcDNSP1 and the truncated mutants were cotransfected, followed by coimmunoprecipitation with either anti-FLAG or anti-His antibody, and immunoblotted with the reciprocal antibody. Results showed that the DNA binding region (amino acids 100 to 300) of p53 coimmunoprecipitated in the presence of NSP1 (Fig. 5B). Input lysates were probed with anti-NSP1 antibody and showed no interaction with p53 domains (Fig. 5C). Input lysates were probed with anti-NSP1 antibody and showed no interaction with p53 domains (Fig. 5D).

MG132, suggesting a role of the proteasomal pathway; thus, to analyze whether NSP1 modulates ubiquitylation of p53, 293T cells were either cotransfected with pcDNSP1 and each of the p53 domains or transfected individually. After 36 h, cell lysates were immunoprecipitated with anti-p53 antibody and probed with anti-FLAG antibody. Results revealed increased ubiquitination of p53 in the presence of NSP1 (Fig. 4C). Input lysates were probed with anti-p53 and anti-NSP1 antibody.

FIG 5 The DNA binding domain (amino acids 100 to 300) of p53 interacts with NSP1. (A) Analysis of expression of truncated constructs of p53. 293T cells were transfected with the region of pFLAG-CMV-p53 from amino acids 1 to 80, pFLAG-CMV-p53 from amino acids 100 to 300, or pFLAG-CMV-p53 from amino acids 300 to 393, followed by immunoblotting with anti-FLAG and anti-β-actin antibody. (B and C) The region from amino acids 100 to 300 interacts with NSP1. 293T cells were either cotransfected with pcDNSP1 and each of the p53 domains or transfected individually. After 36 h, cell lysates were immunoprecipitated with anti-p53 antibody and probed with anti-FLAG antibody. Whole-cell lysates were immunoblotted with anti-NSP1 and anti-FLAG antibody. (D and E) The DNA binding domain of p53 interacts with NSP1 in vitro. Different IVT products of p53 mutants or IVT NSP1 were immobilized with Ni⁺², and enterokinase-treated reciprocal IVT proteins were incubated with them, followed by immunoblot analysis of the proteins interacting with the immobilized proteins.
using an in vitro transcription-translation system to analyze the interaction between the NSP1 and p53 mutants in vitro. Consistent with previous results (Fig. 5B and C), only the DNA binding core domain (p53 [amino acids 100 to 300]) interacted with NSP1 (Fig. 5D and E).

The RING domain of NSP1 is required for p53 degradation but not for interaction. The N-terminal ring domain of NSP1 is presumed to contain ubiquitin ligase activity (21). To know whether this region is necessary for p53 binding and degradation, only the RING domain (amino acids 1 to 82; RING-NSP1) and RING domain mutant NSP1 (amino acids 83 to 482) cloned in pcDNA. (B) Ectopic expression of fragments of NSP1. 293T cells were transfected with pcDRING-NSP1 and pcΔRING-NSP1 for 36 h, followed by immunoblotting with anti-His and anti-β-actin antibody. (C and D) The RING domain is not necessary for interaction with p53. 293T cells were either transfected with pcCMV-p53, pcDRING-NSP1, or pcΔRING-NSP1 individually or cotransfected with either pcCMV-p53 and pcDRING-NSP1 or pcCMV-p53 and pcΔRING-NSP1 separately for 36 h and coimmunoprecipitated with either anti-His (C) or anti-p53 (D) antibody, followed by immunoblotting with the reciprocal antibody. Whole-cell lysates were immunoblotted with anti-p53 anti-His antibody. (E) The RING domain is necessary for degradation of p53. 293T cells were either transfected with pcCMV-p53 or cotransfected with either pcCMV-p53 and pcDNSP1 or pcCMV-p53 and pcΔRING-NSP1 separately for 36 h, followed by immunoblotting with anti-p53 and anti-β-actin antibody. (F) The RING domain is necessary for ubiquitination of p53. 293T cells were either transfected with pcCMV-p53 or cotransfected with pcCMV-p53, pcΔRING-NSP1, and pFLAG-CMV-Ub or pcCMV-p53, pcDNSP1, and pFLAG-CMV-Ub for 36 h, followed by coimmunoprecipitation with anti-p53 antibody and immunoblotting with anti-FLAG antibody.

FIG 6 The RING domain of NSP1 is necessary for ubiquitinylation and degradation, but it does not bind to p53. (A) Schematic representation of NSP1, the RING domain (amino acids 1 to 82) of NSP1, and RING domain mutant NSP1 (amino acids 83 to 482) cloned in pcDNA. (B) Ectopic expression of fragments of NSP1. 293T cells were transfected with pcDRING-NSP1 and pcΔRING-NSP1 for 36 h, followed by immunoblotting with anti-His and anti-β-actin antibody. (C and D) The RING domain is not necessary for interaction with p53. 293T cells were either transfected with pcCMV-p53, pcDRING-NSP1, or pcΔRING-NSP1 individually or cotransfected with either pcCMV-p53 and pcDRING-NSP1 or pcCMV-p53 and pcΔRING-NSP1 separately for 36 h and coimmunoprecipitated with either anti-His (C) or anti-p53 (D) antibody, followed by immunoblotting with the reciprocal antibody. Whole-cell lysates were immunoblotted with anti-p53 anti-His antibody. (E) The RING domain is necessary for degradation of p53. 293T cells were either transfected with pcCMV-p53 or cotransfected with either pcCMV-p53 and pcDNSP1 or pcCMV-p53 and pcΔRING-NSP1 separately for 36 h, followed by immunoblotting with anti-p53 and anti-β-actin antibody. (F) The RING domain is necessary for ubiquitinylation of p53. 293T cells were either transfected with pcCMV-p53 or cotransfected with pcCMV-p53, pcΔRING-NSP1, and pFLAG-CMV-Ub or pcCMV-p53, pcDNSP1, and pFLAG-CMV-Ub for 36 h, followed by coimmunoprecipitation with anti-p53 antibody and immunoblotting with anti-FLAG antibody.

The NSP1-mediated degradation of p53 delays p53-mediated apoptosis induction during SA11 infection. Induction of apoptosis is a physiological function of p53, which is controlled by its two downstream proteins, PUMA and Bax (33). To know the effect of p53 degradation on PUMA expression and Bax activation during infection, mitochondrial fractions or whole-cell lysates from MA104 cells infected with SA11 (0 to 14 h) were analyzed by immunoblotting. Consistent with the decreased p53 levels during early infection, depletion of PUMA was also observed until 8 hpi and Bax translocation to mitochondria was observed only after 10 hpi (Fig. 7A). Similar results were observed in cells infected with...
another rotavirus strain, A5-13 (see Fig. S3 in the supplemental material). However, in A5-16 (NSP1 mutant)-infected cells, early PUMA upregulation (2 hpi) and translocation of Bax to mitochondria (4 hpi) were observed (Fig. 7B). To confirm whether modulation of PUMA and Bax during rotavirus infection is PI3K dependent or not, MA104 cells were infected with either SA11 (A) at an MOI of 3 or A5-16 (B) at an MOI of 3 or kept mock infected for the indicated time points, followed by immunoblot analysis of either the whole-cell lysates with anti-PUMA and anti-β-actin antibody or the mitochondrial fraction with anti-Bax and anti-Cox4 antibody. (C and D) p53-induced modulation of apoptosis during SA11 (NSP1 positive) or A5-16 (NSP1 negative) infection is independent of NSP1-mediated PI3K activation. MA104 cells were infected with either SA11 (C) or A5-16 (D) at an MOI of 3 in the presence of LY294002 for the indicated time points, followed by immunoblot analysis of either the whole-cell lysates with anti-PUMA and anti-β-actin antibody or the mitochondrial fraction with anti-Bax and anti-Cox4 antibody. (E) NSP1 counteracts the proapoptotic function of p53. 293T cells were either transfected with pCMV-p53, cotransfected with either pcDNSP1 and pCMV-p53 or pCMV-p53-mt135 and pcDNSP1, or kept mock transfected for 36 h, followed by immunoblot analysis of the whole-cell lysate (PUMA, caspase-9, caspase-3, PARP, and β-actin antibodies) and the mitochondrial fraction (anti-Bax, and anti-Cox4 antibodies).

**DISCUSSION**

The innate immune response against virus infection is the first line of defense in vertebrates, and viruses have also developed strategies to evade the early immune response for efficient infection (35, 36). p53 has a potential role in antiviral innate immunity, as it induces the apoptotic machinery as well as transactivates several genes which are directly involved in viral sensing, cytokine production, and inflammation (7). Similarly viruses have been shown to counteract p53, as reported in hepatitis B virus (37), Epstein-Barr virus (38), human papillomavirus (39), etc. In this study, we
have shown the mechanism by which rotavirus modulates the p53 level during different stages of infection for its own advantage.

During the early stages of rotavirus infection, when replication is the principal focus of the virus, p53 downregulation was observed irrespective of the strain type (Fig. 1A; see Fig. S1 in the supplemental material). Recent studies have shown that transcription of the p53 gene depends on the interferon response (26) and during infection interferon induction is downregulated by rotaviruses (20). However, during rotavirus infection, p53 regulation seems to be an interferon-independent phenomenon, since p53 was also downregulated in rotavirus-infected Vero cells, which lack a functional interferon pathway (Fig. 1C).

NSP1’s role in p53 downregulation was predicted when no p53 degradation was observed in A5-16 (NSP1 mutant)-infected cells (Fig. 2A). Moreover, in the absence of viral infection, p53 was also degraded in NSP1-overexpressing cells (Fig. 2B). No significant change in p53 transcript levels (Fig. 1D) and restoration of the p53 levels in the presence of MG132 suggested posttranslational proteasomal degradation of p53 during rotavirus infection (Fig. 2C).

In addition to modulation of the interferon response (20), NSP1 has also been shown to activate the PI3K/AKT pathway (19). AKT has been shown to regulate the phosphorylation of MDM2, which, when activated, induces p53 degradation (29). Results revealed that the p53-modulating activity of NSP1 was independent of its PI3K/AKT activation ability, since in the presence of both PI3K inhibitor (LY294002) and MDM2 inhibitor (Nutlin-3), SA11-induced p53 degradation was observed (Fig. 3A to D). No effect of LY294002 on viral gene (NSP1) expression was observed (Fig. 3A), suggesting that it does not directly affect viral replication. In our previous study (19), the lower viral titers observed in the presence of LY294002 could be due to premature apoptosis initiation, resulting in reduced numbers of infectious viral particles.

The versatility of the ubiquitin-proteasome system in controlling cellular homeostasis through posttranslational protein modification makes it an attractive target for viruses. Viruses manipulate this system by diverse mechanisms. Baculoviruses encode their own ubiquitin (40), and modified cellular ubiquitin has also been found in poxviruses and herpes simplex viruses (41). Some viruses encode their own E3 ligase (42), and others recruit cellular E3 ligase for directing the ubiquitin system to their choice of substrate (43). E3 ligase carries out the final step of substrate binding and ubiquitinylation of it (44). Like most of the virus-encoded E3 ligases, NSP1 has a RING domain in the N terminus (amino acids 1 to 82) (21). Among the three types of ubiquitin ligases, the RING family is the largest one (45). Members of this family of ubiquitin ligases facilitate ubiquitinylation by binding charged ubiquitin-conjugating enzymes (E2) and substrate proteins simultaneously without being covalently attached to ubiquitin. NSP1 was found to communoprecipitate with p53 in both cell lysates (Fig. 4A) and under in vitro conditions (Fig. 4B), and the amount of ubiquitini-
ylation of p53 is increased in the presence of NSP1 (Fig. 4C), confirming the specific interaction of NSP1 with p53. Interaction of NSP1-p53 during early stages of infection (Fig. 4D and E) correlated with p53 degradation during early infection (Fig. 1A). A coimmunoprecipitation experiment with different p53 and NSP1 mutants identified interaction between the DNA binding core domain (amino acids 100 to 300) of p53 and the C-terminal domain of NSP1 (∆RING NSP1, amino acids 83 to 482) (Fig. 5B to E and 6C and D). The RING domain of E3 ligases interacts not only with E2 but also sometimes with the substrate; however, in the case of NSP1, the RING domain was found to be essential for p53 degradation and ubiquitylation (Fig. 6E and F) but did not interact with p53 (Fig. 6C and D), suggesting that full-length NSP1 is required for modulating p53 function.

p53 integrates multiple stress signals into a series of diverse antiproliferative responses; among them, the ability to activate apoptosis by transcription-dependent and -independent pathways is the most important (46). p53 transactivates several apoptotic genes (bax, puma, bid) (47) and activates the translocation of Bax to mitochondria (48). PUMA plays a vital role in Bax activation and initiation of apoptosis (49). Consistent with previous results, bax and puma transcripts were downregulated at the initial time points of SA11 infection, but expression was restored during the late infection (Fig. 1D). Similarly, increased levels of PUMA protein or translocation of Bax to mitochondria occurred during late hours (10 hpi) of SA11 or A5-13 infection (Fig. 7A; see Fig. S3 in the supplemental material), unlike in NSP1 mutant strain (A5-16)-infected cells, where Bax activation and PUMA upregulation occurred during early infection (2 to 4 hpi) (Fig. 7B). A5-16 was previously reported to poorly activate the PI3K pathway, contributing to induction of early apoptosis (19). Failure to modulate PUMA and Bax could also contribute further in early apoptosis and to the lower growth rate of A5-16 (28). However, in the presence of PI3K inhibitor, no significant effect on PUMA expression or Bax translocation was observed in either SA11- or A5-16-infected cells, suggesting no role of NSP1-mediated PI3K activation in p53-induced apoptosis during rotavirus infection (Fig. 7C and D). Inactivation of p53 by dominant negative p53 resulted in inhibition of rotavirus-induced PUMA upregulation or Bax translocation to mitochondria (Fig. 8A). This, in turn, also resulted in reduced apoptosis induction in DNP53-expressing cells either transfected with pCMV-p53 or infected with SA11 compared to that in pCMV-p53-transfected or SA11-infected cells (Fig. 7E and 8C and D). In the presence of NSP1 with or without PI3K/akt inhibitor, the level of p53-stimulated apoptosis was significantly reduced compared to that in cells only overexpressing p53 (see Fig. S2 in the supplemental material), suggesting the significance of NSP1 in modulation of p53-induced apoptosis during rotavirus infection. Since inhibition of apoptosis during early stages of infection is beneficial for the virus to complete its life cycle, activation of the PI3K pathway and degradation of p53 by NSP1 can be a viral strategy for inhibiting apoptosis. In our previous study, we demonstrated that NSP4 plays a crucial role in SA11-induced apoptosis, as it targets mitochondria and destabilizes it, but in the presence of NSP4 small interfering RNA, apoptosis was still observed during late infection (34), which might have been due to increased p53 levels, which in turn activate Bax, during late infection (50). Thus, control of apoptosis through a biphasic pattern of the p53 level is a highly efficient strategy for successful infection by rotavirus. Overall, the results show that NSP1 targets diverse cellular proteins to modulate host innate immune responses according to the requirement of rotavirus during different steps of infection.

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