Rotavirus Controls Activation of the 2′-5′-Oligoadenylate Synthetase/RNase L Pathway Using at Least Two Distinct Mechanisms

Liliana Sánchez-Tacuba, Margarito Rojas, Carlos F. Arias, Susana López
Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

ABSTRACT
The innate immune response is the first line of defense of the host cell against a viral infection. In turn, viruses have evolved a wide variety of strategies to hide from, and to directly antagonize, the host innate immune pathways. One of these pathways is the 2′-5′-oligoadenylate synthetase (OAS)/RNase L pathway. OAS is activated by double-stranded RNA (dsRNA) to produce 2′-5′ oligoadenylates, which are the activators of RNase L; this enzyme degrades viral and cellular RNAs, restricting viral infection. It has been recently found that the carboxy-terminal domain (CTD) of rotavirus VP3 has a 2′-5′-phosphodiesterase (PDE) activity that is able to functionally substitute for the PDE activity of the mouse hepatitis virus ns2 protein. This particular phosphodiesterase cleaves the 2′-5′-phosphodiester bond of the oligoadenylates, antagonizing the OAS/RNase L pathway. However, whether this activity of VP3 is relevant during the replication cycle of rotavirus is not known. Here, we demonstrate that after rotavirus infection the OAS/RNase L complex becomes activated; however, the virus is able to control its activity using at least two distinct mechanisms. A virus–cell interaction that occurs during or before rotavirus endocytosis triggers a signal that prevents the early activation of RNase L, while later on the control is taken by the newly synthesized VP3. Cosilencing the expression of VP3 and RNase L in infected cells yields viral infectious particles at levels similar to those obtained in control infected cells, where no genes were silenced, suggesting that the capping activity of VP3 is not essential for the formation of infectious viral particles.

IMPORTANCE
Rotaviruses represent an important cause of severe gastroenteritis in the young of many animal species, including humans. In this work, we have found that the OAS/RNase L pathway is activated during rotavirus infection, but the virus uses two different strategies to prevent the deleterious effects of this innate immune response of the cell. Early during virus entry, the initial interactions of the viral particle with the cell result in the inhibition of RNase L activity during the first hours of the infection. Later on, once viral proteins are synthesized, the phosphodiesterase activity of VP3 degrades the cellular 2′-5′-oligoadenylates, which are potent activators of RNase L, preventing its activation. This work demonstrates that the OAS/RNase L pathway plays an important role during infection and that the phosphodiesterase activity of VP3 is relevant during the replication cycle of the virus.

Rotaviruses, an important cause of severe gastroenteritis in the young of many animal species, including humans, are nonenveloped viruses formed by a triple-layered capsid surrounding the viral genome composed of 11 segments of double-stranded RNA (dsRNA). After entering the cell, the incoming rotavirus particle is uncoated, losing the outermost-layer proteins, VP4 and VP7, yielding a transcriptionally active double-layered particle (DLP) that consists of VP6 and the core proteins VP2, VP1, and VP3. The viral RNA transcripts encode six structural and six nonstructural proteins (1). These transcripts also serve as the templates for the synthesis of RNA negative strands to form the dsRNA genomic segments. Naked genomic viral dsRNA genome is never exposed in the cytoplasm, since it is transcribed and replicated inside replication intermediate particles by VP1, the viral RNA-dependent RNA polymerase (RdRp), and capped by VP3, the guanylyl- and methyltransferase of the virion (2). The replication of the viral RNA and the initial morphogenesis of the virions take place in viroplasms (perinuclear, electrodense cytoplasmic structures), concurrently with the packaging of RNA transcripts into core-replication intermediate particles (3). This process leads to the production of new, transcriptionally active DLPs that initiate an enhanced second round of transcription (4).

The host response to viral dsRNA is a key component of the interferon (IFN) system and represents the first line of defense of the cells against virus infection. Viruses have evolved different strategies to hide their genetic material from the cell sensors. If detected, however, viruses have also developed a series of countermeasures to prevent the deleterious effects of the antiviral responses of the cell (5, 6). During rotavirus replication, the genomic dsRNA hides from the IFN system within replication intermediates immersed in viroplasms. However, several findings suggest that rotaviral RNAs, most probably highly structured viral transcripts or uncapped viral mRNAs, are exposed to cell sensors at some point during the replication cycle: viral dsRNA has been detected in the cytoplasm of infected cells by a monoclonal antibody (MAb) that recognizes dsRNA stretches longer than 40 bases (7), the RNA sensors RIG-I and MDA5 are activated and mediate the...
IFN response in the rotavirus-infected cell (8, 9), and the dsRNA-dependent kinase PKR is activated and phosphorylates translation factor eIF2α (a subunit of eukaryotic initiation factor 2) (10).

The presence of cytoplasmic dsRNA also triggers the activation of the 2′-5′-oligoadenylate synthetase/RNase L (OAS/RNase L) pathway, which catalyzes the degradation of most RNAs, contributing to a general shutoff of the protein synthesis (11). dsRNA induces the oligomerization of the 2′-5′-oligoadenylate synthetase (OAS), activating it. Active OAS synthesizes 2′-5′-oligoadenylates (2-5 A) by using ATP as a substrate; in turn, 2-5 A bind with high specificity and affinity to monomeric, inactive RNase L, inducing its dimerization and its activation. This RNase cleaves within single-stranded regions of cellular or viral RNA, mainly on the 3’ sides of UpAp and UpUp dinucleotides, leaving 3′-phosphoryl and 5′-hydroxyl groups at the termini of the RNA cleavage products (11).

It was recently reported that the carboxy-terminal domain (CTD) of rotavirus strain SA11 VP3 has a 2′-5′-phosphodiesterase (2-5-PDE) activity, similar to that of the ns2 protein of the coronavirus mouse hepatitis virus (MHV), which antagonizes the antiviral activity of RNase L by degrading 2-5 A (12, 13). By sequence analysis, ns2 and VP3 were predicted to be members of a Lyt-like family of the 2 H phosphodiesterase superfamily. The members of this superfamily are characterized by the presence of 2 conserved catalytic histidine residues and include both viral and cellular proteins of diverse origins (14). It has been shown that the VP3 CTD has PDE activity in vitro and when transiently expressed in mammalian cells. Furthermore, the VP3 CTD is able to functionally substitute for ns2 during MHV infection (13). More recently, the CTD of rotavirus strain rehsv rotavirus (RVR) has been cocryrstallized with its substrate 2-5 A, and its in vitro activity has been demonstrated elsewhere (15). However, the role of this activity of VP3 during rotavirus infection has not been explored.

Since several dsRNA-induced mechanisms are triggered during rotavirus infection, in this work we studied whether the OAS/RNase L pathway is activated in rotavirus-infected cells and also characterized the strategies used by the virus to counteract this cellular defense system. We found that during infection the OAS/RNase L pathway is activated; however, rotavirus is able to counteract this system using at least two mechanisms. The first prevents the early activation of the pathway during virus entry, and in the second mechanism the PDE activity of VP3 plays an important role to control the activation of RNase L.

MATERIALS AND METHODS

Cell culture and viruses. The rhesus kidney epithelial cell line MA104 (ATCC), grown in Dulbecco’s modified Eagle’s medium (DMEM)-reduced serum (Thermo Scientific HyClone, Logan, UT) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Biowest, Kansas City, MO) at 37°C in a 5% CO2 atmosphere, was used for all experiments carried out in this work. The simian rotavirus strain RRV used in this work has been described previously (16) and was propagated in MA104 cells as described previously (17). Prior to infection, rotavirus cell lysates were activated with 10 μg/ml of trypsin (Gibco Life Technologies, Carlsbad, CA) for 30 min at 37°C. For some assays, RRV was inactivated by treatment with psoralen and UV as previously reported (18). Briefly, an RRV lysate was mixed with psoralen (Sigma-Aldrich, St. Louis, MO) to a final concentration of 40 μg/ml and was exposed to a UV lamp for 2 h on ice. To verify the inactivation, the titer of the UV-psoralen-treated virus was determined by an immunoperoxidase focus assay, and the integrity of the viral particles was determined by a hemagglutination assay. Inactivated RRV is here referred to as irRRV.

Reagents and antibodies. The small interfering RNAs (siRNAs) were purchased from GE Healthcare Dharmacon (Lafayette, CO). The sequences of the siRNAs against the rotavirus genes used in this work have been previously reported (10). As an irrelevant control, the siGENOME nontargeting siRNA 3 from Dharmacon was used. A monoclonal antibody (MAB) to RNase L and polyclonal antibodies to OAS1 and OAS3 were obtained from Abcam (Cambridge, MA). Rabbit hyperimmune sera to rotavirus VP1 (anti-VP1), to NSP5 (anti-NSP5), and to a recombinant human vimentin (anti-Vim) were produced in our laboratory. MAB 159 was kindly donated by H. B. Greenberg, Stanford University; Stanford, CA, and MAB HS2 was previously described (19). Horseradish peroxidase-conjugated goat anti-rabbit polyclonal and anti-mouse antibodies were purchased from PerkinElmer Life Sciences (Boston, MA). The 5′-phosphorylated 2′-5′-linked oligoadenylates (2-5 A) were kindly provided by R. Silverman (Cleveland Clinic, Cleveland, OH).

Transfection of siRNAs, poly(I:C), dsRNA, and 2-5 A. Transfection and cotransfection of siRNAs into MA104 cells were performed in 48-well plates using a reverse transfection method as described previously (20). To transfect poly(I:C), dsRNA, or 2-5 A, MA104 cells previously transfected or not with the indicated siRNAs were incubated for 1 h at 37°C with minimum essential medium (MEM) containing Lipofectamine 2000 (26.66 μl/ml; Invitrogen, Carlsbad, CA) and 5 μg/ml of synthetic poly(I:C) (Sigma-Aldrich, St. Louis, MO), 10 μg/ml of purified viral dsRNA, or 2.5 μM 2-5 A. After this time, the transfection mixture was replaced with MEM and the cells were incubated for different times at 37°C before harvesting. None of the siRNAs tested were toxic for the cells, as determined by a lactate dehydrogenase (LDH) release assay, using a commercial kit (Sigma-Aldrich Co., St. Louis, MO).

Radiolabeling of proteins. Cells grown in 48-well plates, transfected or not with siRNAs, were mock infected or infected with rotavirus at a multiplicity of infection (MOI) of 10. At different times postinfection, the medium was replaced by MEM without methionine supplemented with 25 μCi/ml of Easy Tag Express 35S labeling mix (Dupont NEN, Boston, MA) and incubated for 30 min at 37°C. Cells were lysed in Laemmli sample buffer, and the samples were resolved by 6% or 10% SDS-PAGE, followed by autoradiography.

Immunoblot analysis. Cells were lysed in Laemmli sample buffer and denatured by boiling for 5 min. Proteins in cell lysates were separated by 10% SDS-PAGE and transferred to Immobilon NC (Millipore, Sweden) using a Transblot. To quantitate the activity of RNase L, the bands corresponding to RNase L were cut out and resolved on RNA chips using the Eukaryote Total RNA Nano software (version 2.61).

Immunofluorescence analysis. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with primary antibodies diluted 1:100 in blocking buffer for 1 h at room temperature. The cell membranes were blocked with 3% BSA for 30 min. The secondary antibodies were conjugated with Alexa Fluor 488, 546, or 647 (Invitrogen, Carlsbad, CA) and were diluted in blocking buffer. The cells were analyzed using a Leica TCS SP5 Confocal microscope. The confocal images were processed using the LAS AF software (version 4.0).

Immunoprecipitation. The small interfering RNAs (siRNAs) were synthesized at Ambion and purchased from GE Healthcare Dharmacon (Lafayette, CO). The siRNAs were transfected into MA104 cells using the siLentFect transfection reagent (Thermo Scientific) and cultured for 24 h. The cells were then harvested, and the cell lysates were sonicated and clarified by centrifugation at 15,000 × g. The cell lysates were incubated with Protein A–agarose (Sigma-Aldrich) at 4°C overnight. The beads were washed extensively and then boiled in sodium dodecyl sulfate sample buffer. The proteins were resolved on 10% SDS-PAGE, transferred to Immobilon NC (Millipore), and probed with specific antibodies against RNase L.

rRNA cleavage assays. Total cellular RNA was isolated at different times postinfection using the PureLink RNA minikit (Ambion-Life Technologies, Carlsbad, CA). RNA was quantitated using a NanoDrop analyzer, and equal amounts of RNA were loaded and resolved on RNA chips (Agilent Technologies, Santa Clara, CA) using an Agilent 2100 Bioanalyzer. To quantitate the activity of RNase L, the bands corresponding to 18S and 28S rRNAs and their degradation products were calculated using the Eukaryote Total RNA Nano software (version 2.61).

Triple-layered particles (TLPs), DLPs, spikeless particles, and dsRNA purification. MA104 cells grown in 150-cm2 flasks were infected as described previously and were harvested 14 h postinfection (hpi), the viral lysates were freeze-thawed three times, and viral particles were concentrated by ultracentrifugation for 1 h at 30,000 rpm at 4°C in an SW40 rotor (Beckman, Fullerton, CA). To obtain spikeless particles, cells were
transfected with an siRNA directed to VP4 (21) for 72 h before rotavirus infection.

The viral pellets were resuspended in TNC buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 10 mM CaCl₂), sonicated three times for 20 s, extracted with Genetron (trichloromonofluorothane), and used to obtain either genomic viral dsRNA or viral particles. To obtain genomic dsRNA, the virus suspension was pelleted through a 40% sucrose cushion by centrifugation for 1 h at 30,000 rpm at 4°C in a SW40 rotor, and viral dsRNA was isolated from the pellet by extraction with phenol-chloroform.

To purify viral particles (DLPs, TLPs, and spikeless particles), CsCl was added to the aqueous phase obtained from the Genetron extraction to obtain a density of 1.36 g/cm³, the mixture was centrifuged for 18 h at 100,000 g, and the viral pellets were resuspended in TNC buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 5 mM EGTA) to remove the remaining outer layer proteins of the particles, and the viral particles were pelleted as described above.

Lipofection of DLPs and spikeless particles. Confluent cells were transfected with DLPs or spikeless particles using Lipofectamine (Invitrogen, Carlsbad, CA) as previously described (7). Briefly, DLPs (10 µg/ml) or spikeless particles (15 µg/ml) were diluted in MEM and incubated with a mixture of Lipofectamine in MEM for 20 min at room temperature. One hundred microliters of this mixture was added to the cells for 1 h at 37°C, and then cells were washed with MEM. Cells were lysed at different times postlipofection.

Northern blotting. Total cellular RNA was isolated using TRIzol reagent (Ambion-Life Technologies, Carlsbad, CA) as previously described (7). Briefly, DLs (10 µg/ml) or spikeless particles (15 µg/ml) were diluted in MEM and incubated with a mixture of Lipofectamine in MEM for 20 min at room temperature. One hundred microliters of this mixture was added to the cells for 1 h at 37°C, and then cells were washed with MEM. Cells were lysed at different times postlipofection.

RESULTS

The OAS/RNase L pathway becomes active during rotavirus infection. To determine if the OAS/RNase L pathway plays a role during the infection cycle of rotaviruses, we silenced the expression of OAS1, OAS3, and RNase L by RNA interference (RNAi) and characterized the effect of their knockdown on viral infectivity. We found that while transfection of the cells with poly(I:C), which is a well-characterized inducer of the OAS/RNase L pathway during the infection process, we evaluated the integrity of rRNAs in cells infected with RRV at different MOIs, compared with that of mock-infected cells, by RNA chip analysis using an Agilent Bioanalyzer. As a control for activation, we transfected the cells with poly(I:C), which is a well-characterized inducer of the OAS/RNase L pathway. We found that while transfection of cells with poly(I:C) induced a clear degradation of the 18S and 28S rRNAs, no rRNA degradation was observed in cells that were infected with the simian rotavirus strain RRV. Twelve hours postinfection, cells were lysed, the amount of infectious virus produced was determined by an immunoperoxidase focus-forming assay (Fig. 1A), and the effectiveness of the silencing was assessed by Western blotting (Fig. 1B). We found that while silencing the expression of OAS3 did not significantly affect the infectivity of RRV, the knockdown of OAS1 and RNase L resulted in about a 2-fold increase in viral infectivity, suggesting that this pathway has a slight deleterious effect during rotavirus infection.

The activation of the OAS/RNase L pathway has as a consequence the degradation of RNA, which can be assessed by the integrity of rRNAs (22). To characterize the level of activation of this pathway during the infection process, we evaluated the integrity of rRNAs in cells infected with RRV at different MOIs, compared with that of mock-infected cells, by RNA chip analysis using an Agilent Bioanalyzer. As a control for activation, we transfected the cells with poly(I:C), which is a well-characterized inducer of the OAS/RNase L pathway. We found that while transfection of cells with poly(I:C) induced a clear degradation of the 18S and 28S rRNAs, no rRNA degradation was observed in cells that were infected with different MOIs of rotavirus RRV (Fig. 2A) or harvested at different times postinfection (Fig. 2B).
The RNA degradation triggered by dsRNA or 2-5 A is prevented during rotavirus infection. We next explored if the lack of degradation of rRNA observed in rotavirus-infected cells was due to a lack of activation of the pathway or to an inhibition of the activity of RNase L. For this, mock-infected or RRV-infected MA104 cells were transfected with either purified viral dsRNA or 2-5 A, and at 9 hpi total RNA was extracted and analyzed for its integrity as indicated above. We found that while there was a clear degradation of rRNAs in cells transfected with either dsRNA or 2-5 A, when the transfected cells were infected with rotavirus at the same time, the degradation of rRNAs was prevented (Fig. 3A and B). These results suggest that the degradation of rRNAs induced by the OAS/RNase L pathway appears to be prevented during RRV infection. The observation that the degradation of rRNAs was prevented when 2-5 A were used suggests that the control exerted by the virus infection was at the level of preventing the activation of RNase L.

Rotavirus infection counteracts the OAS/RNase L pathway by at least two different mechanisms. To characterize the mechanism through which rotavirus prevents the activation of the OAS/RNase L pathway, we first studied if the entry of the virus had a role in the control of the pathway. To achieve this, cells were either infected with dsRNA or 2-5 A, or transfected with double-layered particles (DLPs) or transfected with double-layered particles (TLPs) that lack the outer layer of the virus and are unable to enter the cells but when introduced into the cytosol by lipofection are competent to start a productive virus replication cycle. At the same time, cells were transfected or not with dsRNA and were then harvested at different times to determine the integrity of the rRNAs. Figure 4A shows that when the cells were infected with TLPs and either treated or not with dsRNA, no degradation of RNA was detected; in contrast, when the cells were only transfected with dsRNA, there was a time-dependent increase in the amount of rRNA degradation observed. When DLPs were transfected into cells, we found that there was a limited degradation of rRNA that reached a plateau between 3 and 6 hpi. When cells were lipofected with DLPs and with dsRNA, we found that the amount of rRNA degraded was larger and, similarly, it reached a plateau between 3 and 6 hpi, although the level of degradation achieved was less than half of that obtained when dsRNA was transfected alone. To discard the possibility that the lipofectant reagent was causing this effect, purified TLPs were mixed with Lipofectamine and used to infect cells that were transfected or not with dsRNA. In these assays, we found that there was no noticeable degradation of rRNA at any time postinfection in the presence or absence of dsRNA (results not shown), indicating that the effect observed with DLPs was not due to the lipofectant. These results suggest that the entry process of the virus might trigger a signal that prevents the early activation of the OAS/RNase L pathway. In addition, a second mechanism activated at a later stage of virus replication to control the rRNA degradation (between 3 and 6 hpi) seems to be operational.

To test whether the second step of control of rRNA degradation induced by rotavirus infection was mediated by a viral product synthesized in infected cells, cells were infected with viral particles inactivated by treatment with psoralen-UV and then transfected or not with dsRNA. These viral particles are able to bind and enter the cells, but they are unable to initiate the transcription of viral mRNAs. We found that there was no degradation of rRNA during the first 3 hpi in cells infected with UV-inactivated virions and transfected with dsRNA, but thereafter, the degradation of rRNA increased with time (Fig. 4B). Taken together, these results suggest that the entry of viral particles triggers a signal that prevents the initial degradation of rRNA, and later on, the virus has a second mechanism, which depends on the de novo synthesis of a viral component, to keep the RNase L inactive.

The outer layer proteins are involved in controlling the activation of RNase L. Since DLPs were unable to prevent the initial activation of RNase L, we tested whether VP4, VP7, or both were responsible for this initial control. To establish the role of VP4, we characterized the effect of transfecting spikeless particles (containing three layers of proteins formed by VP2, VP6, and VP7 but not VP4) on the degradation of rRNA. These particles were puri-
fied from RRV-infected cells in which the expression of VP4 was silenced by RNAi (Fig. 5A, inset). We have previously shown that these particles, like DLPs, are able to establish a productive replication cycle once they are introduced into the cells by lipofection (21). We found that when these particles were transfected into MA104 cells, a limited degradation of rRNAs was observed, which seemed to reach a plateau at 3 hpi. When these particles were transfected together with dsRNA, an increased degradation of rRNA was observed, but it also reached a plateau between 3 and 6 hpi (Fig. 5A). Interestingly, the spikeless particles behaved similarly to DLPs, suggesting that the absence of VP4 in these particles mimics the behavior of the absence of both surface layer proteins.

In a different approach to evaluating the role that the internalization of the virus might have in the early control of the activity of

FIG 4 Rotavirus infection counteracts the OAS/RNase L pathway in at least two different steps. (A) MA104 cells were either infected with CsCl-purified TLPs or transfected with 10 μg/ml of DLPs. At the same time, the cells were transfected or not with 10 μg/ml of purified rotavirus dsRNA (dsRNA). At the indicated times, the cells were harvested and total RNA was purified and analyzed on a Bioanalyzer. (B) MA104 cells were infected either with RRV (MOI of 10) or with UV-psoralen-inactivated (iRRV) rotavirus and at the same time transfected or not with dsRNA (10 μg/ml). Cells were harvested at the indicated times, and total RNA was extracted and analyzed on a Bioanalyzer. The cleavage products of RNase L were quantitated under each condition using the Bioanalyzer system as described in Materials and Methods. The arithmetic means ± standard deviations from three independent experiments are shown.

FIG 5 The entry of the viral particle prevents the early activation of RNase L. (A) MA104 cells were transfected with 15 μg/ml of spikeless particles (SIPs), and at the same time, cells were transfected or not with 10 μg/ml of purified rotavirus dsRNA (dsRNA). At the indicated times, the cells were harvested and total RNA was purified and analyzed on a Bioanalyzer. (B) Trypsin-treated RRV (MOI of 10) was incubated with MAb 159, MAb HS2, or PBS (as a control) for 2 h at 37°C. One hundred microliters of virus-antibody mixtures was inoculated onto MA104 cell monolayers in 48-well plates. After the adsorption period, the cells were transfected or not with 10 μg/ml of purified rotavirus dsRNA (dsRNA). At the indicated times, the cells were harvested and total RNA was purified and analyzed on a Bioanalyzer. The cleavage products of RNase L were quantitated under each condition using the Bioanalyzer system as described in Materials and Methods. The arithmetic means ± standard deviations from three independent experiments are shown. (Inset) To evaluate the efficiency of neutralization of MAb 159, MA104 cells were mock infected or infected with the same virus-antibody mixtures, and at 8.5 hpi, cells were labeled for 30 min with 25 μCi/ml of Easy Tag Express 35S and then lysed. The labeled proteins were resolved by SDS-10% PAGE and detected by autoradiography.
RNase L, we used MAb 159, a neutralizing antibody that binds to trimeric RRV VP7 and prevents the disassembly of the viral particles but not their endocytosis (23). We found that in contrast with viral particles that were preincubated with MAb HS2, which binds to VP5 but does not neutralize viral infectivity (Fig. 5B, inset), the addition to cells of MAb 159-preincubated particles prevented rRNA degradation in the initial 3 hpi, but thereafter, an increased rRNA degradation was observed (Fig. 5B), resembling the behavior of the UV-inactivated viral particles. Taken together, these results suggest that the initial control of the degradation of rRNA is mediated by an interaction of the virus particle with a cell component that occurs during or prior to virus cell internalization.

**VP3 is needed to prevent the degradation of rRNAs.** As previously mentioned, it has been found that the CTD of VP3 has PDE activity and that this domain is able to replace the PDE activity of other known cellular and viral phosphodiesterases. To establish whether this activity is relevant for rotavirus replication and whether VP3 is the only viral protein involved in the control of the OAS/RNase L pathway after virus entry, we silenced the expression of each viral protein using RNAi, and the effect of the absence of each viral protein on rRNA stability was determined. In these assays, cells were transfected with siRNAs directed to each of the structural and nonstructural proteins (not shown) of the virus or with an irrelevant siRNA as a control and 72 h posttransfection were infected with RRV and transfected or not with dsRNA. The knockdown of each protein was verified by analyzing the synthesis of $^{35}$S-labeled proteins during the infection (Fig. 6A, shown only for the structural proteins). To determine the effect of silencing the expression of each protein on the stability of rRNAs, total RNA was extracted from cells that were transfected with each siRNA, infected with RRV, and transfected or not with dsRNA and was analyzed in the Bioanalyzer as previously described. We found that the knockdown of VP3 was the only treatment that had an effect on the degradation of rRNAs, since in the absence of this structural protein there was a clear degradation of rRNAs, while under the other conditions tested the rRNA remained intact (data not shown). Figure 6B shows the results obtained when either VP1 or VP3 was silenced and the cells were infected or not with RRV and treated with 2-5' A, where it can be seen that in the absence of VP3, but not of VP1, there was a significant degradation of rRNAs. These results directly demonstrate that VP3 plays a role in the control of RNase L during the infectious cycle of rotavirus.

**The phosphodiesterase activity of VP3 is essential for viral replication.** VP3 is the guanylyl-methyltransferase of the virus (24, 25). We have previously shown that when the expression of this protein is silenced by RNAi, the synthesis of viral mRNA and dsRNA decreases by about 10-fold, while the synthesis of the other viral proteins is not significantly affected (4); nevertheless, the yield of infectious progeny virus decreases by about 80%. To determine which activity of VP3, its PDE activity or its capping activity, is responsible for the decreased viral yield, we silenced the expression of VP3 and of RNase L simultaneously, with the idea of eliminating the deleterious effect of RNase L on the yield of infectious virus (Fig. 1A) and at the same time discarding the role of the PDE activity of VP3. This assay would allow us to evaluate the role of the capping activity in the replication cycle of the virus. For these assays, cells were transfected with the siRNAs directed to VP1, VP3, or RNase L either individually or in combination, and 72 h posttransfection, cells were infected with RRV. At 12 hpi, cells were harvested and the amount of infectious virus produced was determined. In these experiments, VP1 was silenced as an internal control since the absence of this protein results in a phenotype similar to that observed when VP3 is silenced, i.e., decreased viral RNA synthesis, nonaltered viral protein synthesis, and decreased viral yield (4). The knockdown of VP1 and RNase L was confirmed...
by Western blotting, while that of VP3 was verified by SDS-PAGE and autoradiography of $^{35}$S-labeled proteins, since an antibody to this protein was not available (Fig. 7A). As already observed (Fig. 1), silencing the expression of RNase L results in a 2-fold increase in virus yield, while silencing VP3 and VP1 individually decreased the production of viral progeny by about 85% (as previously shown [4]) (Fig. 7B). When VP3 was silenced at the same time as RNase L, we found that the amount of infectious virus produced under these conditions was even higher than that produced in control cells transfected with an irrelevant siRNA, while this was not the case when silencing the combination of RNase L and VP1, in which the production of infectious virus was not recovered (Fig. 7B), indicating that under conditions where the capping of rotavirus mRNAs does not occur or at least is greatly diminished, rotavirus replication is not affected.

The PDE function of VP3 protects viral RNA from RNase L degradation. To determine if preventing the activation of RNase L was important for the stability of viral transcripts, we decided to look for the integrity of viral mRNAs by a Northern blot assay. In this case, cells were transfected and infected as in the experiments shown in Fig. 7A, and at 9 hpi cells were harvested and total RNA was extracted, subjected to electrophoresis, and transferred to a Hybond membrane. The separated RNAs were hybridized with a $^{32}$P-labeled oligonucleotide complementary to RRV gene 10 and with an oligonucleotide complementary to the 18S rRNA as a loading control (Fig. 7C). We found that when the expression of either VP1 or VP3 was silenced during the infection, there was a significant reduction in the amount of gene 10 mRNA compared to the amount of this gene found when the cells were treated with an irrelevant siRNA. In contrast, the amount of gene 10 almost doubled when the expression of RNase L was silenced, suggesting that this RNase has a deleterious effect on viral transcripts. When VP3 was silenced in combination with RNase L, the amount of mRNA from gene 10 detected was larger than that found when cells were transfected with the irrelevant siRNA, in contrast with the case when VP1 and RNase L were silenced simultaneously, where the amount of gene 10 mRNA detected was negligible. Taken together, these results suggest that the PDE function of VP3 is very important for the stability of viral mRNAs and consequently for replication of the virus, while the capping activity of this protein does not seem to be essential for the production of progeny virus at least in one infectious cycle.

**DISCUSSION**

To successfully replicate, viruses have evolved a series of strategies to counteract the innate immune responses of the host cell (5, 6). Here, we have found that the OAS/RNase L pathway is activated during the replication of rotaviruses in MA104 cells, but the virus is able to control the activity of this RNase L by at least two distinct mechanisms. Early in the infection, during viral entry, there seems...
to be a mechanism by which the initial interactions of the virus with the cell elicit a signal that delays the activation of RNase L. This conclusion is based on the observation that infection with TLPs or with UV-inactivated virions prevents the degradation of rRNAs in the first 3 h of infection. In contrast, this initial control is not observed when the cells are treated with DLPs or spikeless particles, since a partial degradation of rRNAs is observed in the first 3 to 6 hpi and then the RNA degradation reaches a plateau and appears to be controlled. We believe that an initial interaction of the outer surface proteins of the viral particle with a receptor on the cell surface triggers a signaling cascade that results in the inhibition or lack of activation of the RNase L that is already present, albeit nonactive, in the host cell. The elements that form part of this signaling cascade are yet to be uncovered, but it is tempting to suggest that the interactions of the viral particle with any of the molecules that have been found to play a role as rotavirus receptors (namely, sialic acid; gangliosides; integrins α2β1, αvβ3, and αvβ2; the heat shock cognate protein hsc70; or JAM-A) (26) could be the trigger that prevents the early activation of RNase L or the control of its activity. It has been previously found that UV-inactivated virions induce an antiviral signaling through a mechanism similar to that of live virus, and this supports the notion that type I IFN is activated when rotavirus structural components are detected rather than, or in addition to, viral replication (27–29). Our findings suggest that at the same time that the virus induces an early antiviral response of the cell, it also triggers signals that inhibit the activation of RNase L and probably other early antiviral responses of the cell.

Once the synthesis of viral proteins starts, there seems to be a second mechanism of control (between 3 and 6 hpi) of the RNase L activity; the PDE activity of VP3 decreases the 2-5 A, preventing the dimerization and activation of RNase L. TLPs, DLPs, and spikeless particles are competent in this control, while UV-inactivated particles, which prevent the initial degradation of rRNA, and the virus particles neutralized with MAb 159 to VP7, which are unable to enter the cell, no longer control the activity of RNase L, underlining the need for newly synthesized VP3.

Until recently, the main functions attributed to VP3 were its activities as a guanylyltransferase and a methyltransferase responsible for the 5' capping and the 2'-O-methylation of the 5' cap structures of the viral transcripts (24, 25), activities that are not 100% efficient, such that a portion of the viral transcripts synthesized in the cell are noncapped and could be recognized as RNA patterns that activate the immune signaling through the RIG-I signaling receptors (30, 31). The recent finding that the CTD of VP3 contains a 2'-5'phosphodiesterase activity that degrades the activators of RNase L makes this protein a multifunctional enzyme that contributes to the virulence and the innate immune evasion mechanisms of rotaviruses.

To determine the relevance of the phosphodiesterase activity of VP3 compared with its capping function during virus replication, we silenced the expression of VP3 and RNase L simultaneously by RNAi, with the rationale that in the absence of RNase L, whose activity could not be controlled in the absence of VP3. The results obtained from these experiments also demonstrate that uncapped viral mRNA can be encapsidated and replicated and, very interestingly, that it can produce infectious viral progeny. It is certainly necessary to directly show the absence of capped RNA in the newly synthesized virions, but the recent demonstration that the capping activity of VP3 is inefficient (31) supports this hypothesis. Interestingly, this observation could also offer an explanation for the high ratio of physical to infectious particles observed in different preparations of rotavirus, as has been recently reported for alphaviruses (32). The present work illustrates how host cells and viruses have coevolved mechanisms to detect and prevent the activation of defense mechanisms, respectively. At the same time that the cell senses the entry of the viral particle and activates a series of anti-viral measures, rotaviruses have evolved a way to prevent this early activation, until they reach the cytoplasmic compartment and begin to synthesize their proteins, some of which (VP3, NSP1, and NSP3 and maybe others) interact with host proteins and allow the virus to control directly and indirectly the innate immune responses of the cell.

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