A Genetically Engineered Rotavirus NSP2 Phosphorylation Mutant Impaired in Viroplasm Formation and Replication Shows an Early Interaction between vNSP2 and Cellular Lipid Droplets

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ABSTRACT Many RNA viruses replicate in cytoplasmic compartments (virus factories or viroplasms) composed of viral and cellular proteins, but the mechanisms required for their formation remain largely unknown. Rotavirus (RV) replication in viroplasms requires interactions between virus nonstructural proteins NSP2 and NSP5, which are associated with components of lipid droplets (LDs). We previously identified two forms of NSP2 in RV-infected cells, a cytoplasmically dispersed form (dNSP2) and a viroplasm-specific form (vNSP2), which interact with hypophosphorylated and hyperphosphorylated NSP5, respectively, indicating that a coordinated phosphorylation cascade controls viroplasm assembly. The cellular kinase CK1 α phosphorylates NSP2 on serine 313, triggering the localization of vNSP2 to sites of viroplasm assembly and its association with hyperphosphorylated NSP5. Using reverse genetics, we generated a rotavirus with a phosphomimetic NSP2 (S313D) mutation to directly evaluate the role of CK1 α NSP2 phosphorylation in viroplasm formation. Recombinant rotavirus NSP2 S313D (rRV NSP2 S313D) is significantly delayed in viroplasm formation and in virus replication and interferes with wild-type RV replication in coinfection. Taking advantage of the delay in viroplasm formation, the NSP2 phosphomimetic mutant was used as a tool to observe very early events in viroplasm assembly. We show that (i) viroplasm assembly correlates with NSP5 hyperphosphorylation and (ii) vNSP2 S313D colocalizes with RV-induced LDs without NSP5, suggesting that vNSP2 phospho-S313 is sufficient for interacting with LDs and may be the virus factor required for RV-induced LD formation. Further studies with the rRV NSP2 S313D virus are expected to reveal new aspects of viroplasm and LD initiation and assembly.

IMPORTANCE Reverse genetics was used to generate a recombinant rotavirus with a single phosphomimetic mutation in nonstructural protein 2 (NSP2 S313D) that exhibits delayed viroplasm formation, delayed replication, and an interfering phenotype during coinfection with wild-type rotavirus, indicating the importance of this amino acid during virus replication. Exploiting the delay in viroplasm assembly, we found that viroplasm-associated NSP2 colocalizes with rotavirus-induced lipid droplets prior to the accumulation of other rotavirus proteins that are required for viroplasm formation and that NSP5 hyperphosphorylation is required for viroplasm assembly. These data suggest that NSP2 phospho-S313 is sufficient for interaction with lipid droplets and may be the virus factor that induces lipid droplet biogenesis in rotavirus-infected cells. Lipid droplets are cellular organelles critical for the replication of many viral and bacterial pathogens, and thus, understanding the mechanism

Citation Criglar JM, Crawford SE, Zhao B, Smith HG, Stossi F, Estes MK. 2020. A genetically engineered rotavirus NSP2 phosphorylation mutant impaired in viroplasm formation and replication shows an early interaction between vNSP2 and cellular lipid droplets. *J Virol* 94:e00972-20. <https://doi.org/10.1128/JVI.00972-20>.

Editor Susana López, Instituto de Biotecnología/UNAM

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Received 19 May 2020

Accepted 21 May 2020

Accepted manuscript posted online 27 May 2020

Published 16 July 2020

of NSP2-mediated viroplasm/lipid droplet initiation and interaction will lead to new insights into this important host-pathogen interaction.

KEYWORDS CK1 α , NSP2, NSP5, PLIN1, lipid droplet, perilipin, protein phosphorylation, replication, rotavirus, viroplasm

Rotaviruses (RVs) remain a major cause of infantile and childhood severe, dehydrating diarrheal disease in many parts of the world and continue to cause ~130,000 deaths of infants and children <5 years of age annually (1), despite the availability of several vaccines. RV, a double-stranded RNA (dsRNA) virus, replicates its virus genome and assembles nascent virus particles in electron-dense cytoplasmic structures called viroplasms that are composed of both viral and cellular proteins and interact with lipid droplets (LDs). A time course of viroplasm formation and interaction with LDs suggests that viroplasms form early (2 to 4 hours postinfection [hpi]) before recruiting or interacting with LDs (2). LDs are single-membrane cellular organelles that serve as storage for triglycerides. Long considered inert, LDs are now recognized as dynamic structures critical for lipid and energy metabolism and cell signaling (3). Viroplasm formation is an indispensable part of the virus life cycle and requires the association of two RV nonstructural proteins, NSP2 and NSP5 (4–8). Fundamental questions about viroplasm/LD assembly remain, including (i) the nature of the mechanism of NSP2 and NSP5 interaction, and (ii) whether either NSP2 or NSP5 physically interacts with LD components to trigger LD biogenesis.

Little is known about the direct interaction between NSP2 and NSP5 or the mechanism of viroplasm assembly. NSP2 is a 35-kDa protein that exists in both a cytoplasmically dispersed form (dNSP2) and a viroplasm-localized form (vNSP2) (9). Both dNSP2 and vNSP2 are detected in RV-infected cells and in RV-infected cell lysates, but as yet, neither form can be specifically recreated *in vitro*. NSP2 isoforms are currently distinguished by conformation-specific monoclonal antibodies (MAbs) (9). *In vitro*, bacterially expressed and purified NSP2 readily oligomerizes into octamers in solution (10, 11) and demonstrates several enzymatic activities, including nucleoside triphosphatase (NT-Pase) and nucleoside diphosphate (NDP) kinase-like functions, suggesting there is an important role for phosphotransfer in NSP2 activities during virus replication (12–16). We recently described an *in vitro* NSP2 autophosphorylation capability for NSP2 resulting in multiple phosphorylations throughout the NSP2 protein (17). Additionally, we showed that the cellular kinase CK1 α phosphorylates NSP2 on serine 313 (S313) located in the C terminus α -helical tail (C-terminal helix [CTH]; amino acids [aa] 295 to 317) (17). Phosphorylation of S313 appears preferential on vNSP2, being phosphorylated at a rate 40 times more than that seen with dNSP2 (9). The CTH of NSP2 is required for viroplasm formation (9) and was predicted to facilitate the formation of interoctamer NSP2 chains through “domain swapping,” or interaction, with the CTH on neighboring NSP2 octamers (13). Our crystallographic analysis of a recombinant phosphomimetic NSP2 protein (NSP2 S313D) showed that phosphorylation of NSP2 S313 likely catalyzes four interoctamer hydrogen bridges between adjacent CTHs of neighboring octamers (aspartic acid 313_{octamer A}-arginine 287_{octamer B}), providing strong evidence that CK1 α phosphorylation of NSP2 could be the catalyst for viroplasm assembly (17).

NSP5 is a RV O-linked glycoprophosphoprotein that exists in multiple phosphorylated isoforms with molecular weights ranging from 26 to 28 kDa for the hypophosphorylated isoforms and from 32 to 34 kDa for the hyperphosphorylated isoforms. Intriguingly, we found that dNSP2 exclusively associates with hypophosphorylated NSP5, and that vNSP2 associates only with hyperphosphorylated NSP5 (9). Together, our data support a hypothesis for a coordinated and temporally regulated phosphorylation-dependent mechanism for viroplasm assembly and virus replication. This hypothesis is further supported by recent work describing the use of a *trans*-complementing cell line that stably produces wild-type (WT) NSP5 protein, which was used to characterize various NSP5 phosphorylation-deficient recombinant RVs and showed that NSP5 hyperphosphorylation is required for viroplasm assembly (18).

FIG 1 rRV NSP2 S313D virus shows delayed replication compared to rRV WT virus. One-step growth curves (MOI = 10) demonstrate a lag in replication of the rRV NSP2 S313D mutant virus (orange line) compared to the rRV WT virus (blue line). Mutant virus replication rates were significantly lower than WT rates throughout the 48-h assay period. (*, P value of <0.05 , two-way ANOVA).

In this report, we used reverse genetics to derive and characterize a viable phosphomimetic recombinant RV, rRV NSP2 S313D, to investigate the role of NSP2 S313 phosphorylation in viroplasm formation, viroplasm assembly, and in LD biogenesis during RV infection.

RESULTS

Reverse engineering recombinant WT and NSP2 S313D mutant rotaviruses. We previously reported that a recombinant phosphomimetic NSP2 protein (NSP2 S313D) rapidly formed crystals *in vitro* (17). On the basis of this crystallography result, we hypothesized that a RV NSP2 S313D phosphomimetic mutant virus would act as a “gain-of-function” mutant and be able to catalyze the faster assembly of viroplasms and thus enhance virus replication. To test this hypothesis, we generated two recombinant RVs (rRV): (i) a rRV with a wild-type genome (rRV WT) and (ii) a recombinant RV with a single genome mutation in gene segment 8 encoding NSP2 to generate a phosphomimetic NSP2 S313D virus (rRV NSP2 S313D) (19). The rRV WT virus was obtained using the standard 7-day passage described in the protocol published by Kanai et al. (19). Recovery of rRV NSP2 S313D, predicted to be a gain-of-function mutant, was achieved only after an extended incubation of the second passage over a period of 5 weeks of uninterrupted incubation time. Both rRV WT and rRV NSP2 S313D viruses were then amplified to high titer.

The phosphomimetic rRV NSP2 S313D virus is viable but shows delayed replication kinetics and a small-plaque phenotype compared to rRV WT virus. The extended incubation time needed to acquire the rRV NSP2 S313D mutant virus suggested that this virus was not a gain-of-function mutant. To determine the fitness of the rRV NSP2 S313D virus compared to the rRV WT virus, we performed a one-step growth kinetics analysis (Fig. 1). Plaque assay analysis of virus in cells and media collected over the course of 48 h showed that the rRV NSP2 S313D virus had a significant lag in replication throughout the observation period. This lag was most notable in the first 10 h after infection. After 10 h, the rRV NSP2 S313D virus replication rate increased and the virus yield reached a titer closer to, but still significantly lower than, that of the rRV WT virus. We measured mRNA levels in rRV WT virus-infected cells and rRV NSP2 S313D virus-infected cells to rule out differences in transcription rates between the wild-type and mutant viruses and found no significant difference (data not shown). Therefore, the analysis suggested that although the NSP2 S313D mutation chemically or structurally acted as a phosphomimetic *in vitro*, the mutation was detrimental to virus replication in RV-infected cells. Analysis of the plates used to titer the virus showed a dramatically different plaque phenotype for the rRV NSP2 S313D virus. While rRV WT virus showed a normal plaque morphology, rRV NSP2 S313D had a smaller plaque size that required

FIG 2 rRV NSP2 S313D virus has a smaller plaque phenotype than that of rRV WT virus. Plaque assay wells were overlaid with neutral red viable stain at 48 h postinfection, and plaques were counted at 72 h postinfection. Visualization of plaques shows that rRV NSP2 S313D (right panel) produced significantly smaller plaques than the rRV WT virus (left panel).

3 days of growth to be detected (Fig. 2), suggesting a replication defect that is consistent with the growth kinetics data.

rRV NSP2 S313D has delayed expression of vNSP2 and NSP5, and NSP5 shows delayed hyperphosphorylation. To determine whether the replication defect in the rRV NSP2 S313D virus was due to delayed or altered viral protein expression, Western blotting (WB) was performed to detect dNSP2, vNSP2, and NSP5 proteins up to the 24-h time point, when replication of both viruses was nearing maximum rates. Although cells were infected with equal concentrations of infectious virus, the appearance of both dNSP2 and vNSP2 (Fig. 3) was delayed in cells infected with the rRV NSP2 S313D mutant virus. However, the delay in the appearance of vNSP2 was more pronounced. Similarly, detection of NSP5 was delayed in cells infected with the rRV NSP2 S313D mutant virus, and most notably, the hyperphosphorylated isoforms of NSP5, particularly the 28-kDa band, were detected later than in the rRV WT virus-infected cells (Fig. 3). Since any mutation in a nonstructural protein is not expected to interfere with virus entry, these results suggested that the delay in virus replication might be due to the somewhat slower formation of vNSP2 and, in particular, to the delay in the formation of the hyperphosphorylated NSP5 isoforms.

Viroplasm assembly is delayed in cells infected with rRV NSP2 S313D mutant virus. Considering that viroplasm formation is dependent on coexpression of NSP2 and NSP5 and that we established delayed detection of these proteins for the rRV NSP2 S313D virus, we hypothesized that viroplasm assembly would also be delayed. To test this hypothesis, we used confocal microscopy and immunofluorescence (IF) and our unique in-house-generated MAb against vNSP2 (9). We assessed the overall appearance and localization of vNSP2 relative to viroplasm formation in rRV WT virus-infected cells and in rRV NSP2 S313D virus-infected cells using the same microscope acquisition settings as is the standard procedure in comparing experimental conditions using microscopy. In the first 12 h, we observed a lower number of infected cells and delayed detection of vNSP2 under the rRV NSP2 S313D virus-infected conditions compared to that for the rRV WT virus (Fig. 4A and B). However, in keeping with the results described above, after 12 h the mutant-infected cells more closely resembled recombinant wild-type (rWT) virus-infected cells.

At 8 h postinfection (hpi), we had already determined by WB that vNSP2 in rRV NSP2 S313D virus-infected cells was detected at a slightly reduced level compared to that in rRV WT virus-infected cells (Fig. 3), but this was not reflected in the imaging results (Fig. 4B). Therefore, we imaged the same cells by changing the imaging conditions to allow detection of lower fluorescence intensity. Under these conditions, we indeed detected mutant vNSP2 D313 protein expression at the earlier time points (Fig. 4B'). This result did not represent background signal from the monoclonal antibody or cellular autofluorescence, as signal was not detected in any of the noninfected cells in the field of

FIG 3 Delayed detection of vNSP2 and hyperphosphorylated NSP5 in cells infected with rRV NSP2 S313D mutant virus. rRV WT virus- and rRV NSP2 S313D virus-infected cell lysates (MOI = 10) were prepared using the same lysis buffer. Identical sample volumes for each gel were aliquoted from the same lysate tubes containing sample buffer with or without reducing agent. Blots were probed with MABs to detect dNSP2, vNSP2, or pAb serum to NSP5. NSP2 probed blots show that the appearance of vNSP2 protein was delayed in cells infected with rRV NSP2 S313D virus compared to cells infected with rRV WT virus (nonreducing gels). Blots probed with antibody against NSP5 show that the higher phosphorylated isoforms of NSP5 appeared later in the cells infected with rRV NSP2 S313D mutant virus than in the cells infected with rRV WT virus (reducing gels). Detection of tubulin run under the reducing conditions was used as a loading control for rRV WT and rRV NSP2 S313D lysate preparations. Apparent molecular weights (MW) are shown on the right side of the WB panels.

view. Surprisingly, vNSP2 D313, at early time points, looks diffuse in the cytoplasm of virus-infected cells, reminiscent of dNSP2, and was not localized into viroplasms, whereas most WT vNSP2 was already concentrated in viroplasms at those times (see insets in Fig. 4A, B, and B'). Thus, we were able to validate the WB data in Fig. 3 and to conclude that the rRV NSP2 S313D virus infects cells just as well as the rRV WT virus and expresses vNSP2 but that vNSP2 D313 has a diffused, not viroplasm-localized, distribution. To further validate this observation, we performed quantitative, unbiased, image analysis at the 8-hpi time point and compared the numbers and sizes of viroplasms, and the cytoplasmic distribution patterns of vNSP2, between the rRV WT and rRV NSP2 S313D virus infections. The analysis showed a decrease in the number (Fig. 4C) and area (Fig. 4D) of viroplasms in the mutant virus-infected cells. Moreover, texture analysis, represented by the heat map in Fig. 4E, was used to highlight the differences in the pattern and appearance of vNSP2 in cells with WT vNSP2 or mutant vNSP2 D313 protein. Texture features are classic metrics used in image analysis to discriminate patterns (i.e., diffuse versus structured) and are evaluated on the basis of correlation matrixes between neighboring sets of pixels. This analysis thus quantified the differences in vNSP2 distribution and characteristics of cells infected with rRV WT virus and rRV NSP2 S313D virus.

In rRV NSP2 S313D virus-infected cells, vNSP2 and NSP5 are dispersed and viroplasm formation is delayed. We next determined the effect of NSP2 D313 protein on early viroplasm formation. We examined viroplasm formation at 5 hpi and show representative images of the infected cells. We first examined the distribution of dNSP2

FIG 4 rRV NSP2 S313D is delayed in viroplasm formation despite the near wild-type level expression of vNSP2 protein. Confocal imaging of MA104 cells infected with either rRV WT or rRV NSP2 S313D virus (MOI = 5) was performed at the indicated times postinfection. Infected cells expressing vNSP2 were detected using Mab 1002 conjugated to anti-mouse-488 secondary antibody. (A and B) Images of rRV WT virus-infected cells (A) compared to rRV NSP2 S313D virus-infected cells (B) appear to show less vNSP2 protein expression in the mutant-infected cells. The images in panels A and B were taken using identical laser intensities and capture parameters. Panel B' shows the same fields as those imaged in panel B but increased laser power was used to demonstrate that vNSP2 S313D protein was expressed in the rRV NSP2 S313D virus-infected cells at early times postinfection, consistent with the WB data (Fig. 3). (Insets) Magnified images of the areas outlined in panels A, B, and B' taken at 8 h postinfection (white dashed boxes) show the dispersed phenotype of vNSP2 S313D in the NSP2 mutant virus-infected cells compared to the punctate viroplasm observed in rRV WT-infected cells, explaining why vNSP2 S313D must be imaged using different confocal laser settings to detect the more diffuse signal. Scale bars in main panels = 20 μ m; scale bars in insets = 10 μ m. (C to E) Image analysis using CellProfiler 2.2 software was used to quantify the numbers of viroplasm/cell (C) and the viroplasm areas (D) and to create a heat map comparison of the texture features (characteristic patterns) of the total vNSP2 protein signal in the cytoplasm at the 8-hpi time point (E) comparing rRV WT and rRV NSP2 S313D mutant virus, where WT values were set as 1. *, $P < 0.05$ (using unpaired t test with Welch's correction; >100 cells/condition were analyzed).

in both rRV WT virus-infected cells and rRV NSP2 S313D virus-infected cells and found no discernible difference in the cytoplasmic distribution of dNSP2 with either virus (Fig. 5A). However, it was readily apparent that the normal distribution of NSP5 was very different in the mutant virus-infected cells. In rRV NSP2 S313D virus-infected cells, we detected NSP5 in numerous small puncta with a concomitant increase in the cytoplasmically diffused fraction of NSP5 compared to the larger viroplasm in the rRV WT virus-infected cells. Probing for vNSP2, we found that the viroplasm-localized form of NSP2 was affected by the single S313D mutation (Fig. 5B). Compared to the normal distribution of vNSP2 observed in rRV WT virus-infected cells, the vNSP2 in the rRV NSP2 S313D mutant virus-infected cells was found to be widely dispersed and more reminiscent of dNSP2 than of wild-type vNSP2. These data suggest that the NSP2 S313D mutation is, in fact, detrimental to the replication of the virus due to impaired viroplasm formation.

FIG 5 The NSP2 S313D mutation results in vNSP2 with a dispersed phenotype and disrupted viroplasm formation. (A) dNSP2 (red) in rRV NSP2 S313D virus-infected cells shows a normal dispersed distribution; however, NSP5 (green) is, uncharacteristically, also dispersed. (B) By comparison, vNSP2 (red) in rRV NSP2 S313D virus-infected cells exhibits a “dispersed” phenotype reminiscent of dNSP2. Data represent results of confocal IF performed with MA104 cells (MOI = 5; 5 hpi). Scale bar = 5 μ m.

Viroplasms form but are disorganized in rRV NSP2 S313D virus-infected cells.

The delayed formation of viroplasms by the rRV NSP2 S313D virus does not appear to represent a failure in protein expression. Despite the lower expression levels of NSP2 and NSP5 in the rRV NSP2 S313D virus-infected cells detected by WB in the first 8 h of the infection (Fig. 3), between 8 and 12 hpi there was significant protein expression that was comparable to rRV WT virus levels. Therefore, to assess the integrity of viroplasms, we analyzed RV-infected cells by transmission electron microscopy (TEM) at 8 hpi, the time point when the replication kinetics are most disparate between the two viruses. RV viroplasms have been well characterized by EM/TEM for decades. They are described as electron-dense, protein-rich structures. Immature double-layered RV particles (DLPs) are often seen at the periphery of viroplasms. These DLPs interact with the viral nonstructural protein NSP4, which is detected surrounding viroplasms. Interaction of immature DLPs with NSP4 triggers the particles to bud into membranes adjacent to viroplasms, where they become transiently enveloped. By an unknown mechanism, the membrane envelope is removed and the outer capsid proteins, VP4 and VP7, are assembled onto the particle to form triple-layered particles. In line with previous EM images of viroplasms (20, 21), we found that the viroplasms in the rRV WT virus-infected cells were morphologically similar (Fig. 6A, left). The representative viroplasm was seen as a dense structure with the associated membrane that enclosed enveloped and nonenveloped virus particles. In contrast, viroplasms in the rRV NSP2 S313D mutant

FIG 6 EM of rRV WT and rRV NSP2 S313D. (A) (Left) EM of MA104 cells infected with rRV WT virus shows a prototypical viroplasm that is electron dense and adjacent to membranes into which newly assembled DLPs bud to complete assembly of the outer capsid proteins. (Right) EM of MA104 cells infected with rRV NSP2 S313D virus shows that NSP2 mutation resulted in loosely assembled viroplasms. (B) Close-up of a rRV NSP2 S313D virus viroplasm showing the loosely packed structure with membranous-like looping in the interior and virus particles associated at the periphery. (C) Enumeration of the disordered versus ordered viroplasms between the two recombinant viruses. (D) (Left) The majority of viroplasms in rRV NSP2 S313D virus-infected cells at 24 hpi resemble viroplasms formed in rRV WT virus-infected cells; however, some viroplasms still appear abnormal. (Right) Magnification of partial WT-like viroplasm and a seemingly unproductive disorganized viroplasm (*).

virus-infected cells were very hard to find and required visualization of the viroplasm-associated membranes that contain virus particles to confirm the identity of the structure. Detection of viroplasms in rRV NSP2 S313D virus-infected cells showed that they were loosely packed and disorganized (Fig. 6A, right), correlating with the light microscopy diffuse cytoplasmic signal described above. Magnified views of some of the rRV NSP2 S313D virus viroplasms showed loose assembly of string-like or membrane-like material with emerging virus particles (Fig. 6B). Comparisons of similar numbers of viroplasms in the WT and mutant viruses showed that the vast majority of viroplasms (90%) in the rRV NSP2 S313D mutant-infected cells were disorganized compared to rRV

FIG 7 The NSP2 S313D protein mutation interferes with rRV WT virus replication. (A) Plaque assay titers from the yield of 8-h coinfections with rRV NSP2 S313D virus and rRV WT virus demonstrate a decrease in rRV WT virus yields (in PFU/ml) at 48 hpi compared to single infections. Results are from replicate wells of two independent experiments. Significance was determined using one-way analysis of variance (ANOVA), with Dunnett's multiple-comparison test for comparisons to the WT rRV control (*, $P < 0.05$; **, $P < 0.01$). (B) The plaque assay plates shown in panel A were allowed to incubate for an additional day to demonstrate that, as expected, no mutant virus plaques from the rRV NSP2 S313D virus contributed to the results of the analysis of panel A. Data represent averages of the number of plaques from replicate wells of one experiment.

WT virus viroplasms (15%) (Fig. 6C). Because the rRV NSP2 S313D viroplasms were difficult to find, even at 8 hpi, and because when they were detected they were found to be loosely organized, our data suggest that the defect in the replication of the rRV NSP2 S313D virus is in viroplasm assembly due to NSP2 and NSP5 posttranslational modifications and not to protein expression. In agreement with the growth curve kinetics seen at 24 hpi, when replication of the rRV NSP2 S313D virus began to approach the levels seen with the WT virus, many more WT-like dense rRV NSP2 S313D viroplasms were detected by EM (Fig. 6D).

NSP2 D313 protein interferes with WT virus replication. We noted that the delay in viral replication was a unique phenotype of the rRV NSP2 S313D virus. Given that the active form of NSP2 assembles into octamers *in vitro* (22), it is possible that the NSP2 D313 protein monomers would be able to interact with WT NSP2 S313 monomers and negatively affect replication of rRV WT virus by interfering with the functions of nonmutant NSP2. To test this, we performed mixed infections with rRV NSP2 S313D and rRV WT viruses and assessed the replication of the WT virus via plaque assay to determine titer. By taking advantage of the delay in mutant virus plaque formation (72 h) compared to WT virus plaque formation (48 h), we were able to determine if there was any effect of coexpression of NSP2 D313 protein with WT NSP2 S313. Coinfection with rRV WT and rRV NSP2 S313D viruses at equal multiplicities of infection (MOI) resulted in a significantly lower virus yield of WT virus than of rRV WT virus alone at 48 hpi (Fig. 7A). Coinfection of rRV WT virus (MOI = 10) with rRV NSP2 S313D virus (MOI = 20) further reduced the WT yield. This shows that rRV NSP2 S313D virus interferes with rRV WT replication. As a control, we show that rRV NSP2 S313D virus formed plaques at 72 h postinfection as expected (Fig. 7B). Overall, these results demonstrate that mutant NSP2 S313D protein interferes with rRV WT virus replication, suggesting that the NSP2 S313D mutation functions as a dominant negative.

Lipid droplet-associated protein perilipin colocalizes with RV protein vNSP2 in viroplasms before NSP5. The delay in viroplasm formation for the rRV NSP2 S313D mutant virus suggested that we now had a tool with which to probe early events of viroplasm formation. This is not possible with WT rotaviruses because normal viroplasm formation is extremely rapid and nascent viroplasms can be seen between 2 and 3 hpi, first with dNSP2 and NSP5 association followed quickly by the appearance and colocalization of vNSP2 with NSP5 (9). To determine if the rRV NSP2 S313D virus could be used to probe early events in viroplasm assembly, we chose to investigate viroplasm

FIG 8 vNSP2 and NSP5 colocalized with LD-specific protein phospho-perilipin 1 (P-PLIN1), but rRV NSP2 S313D mutant virus showed that vNSP2 colocalized with LDs before, and prior to association with, NSP5. (A) MA104 cells infected with RV strain SA11 4F virus at 8 hpi. Immunofluorescence imaging shows that viroplasm-specific protein vNSP2 (upper panel, green) colocalized with LD-specific protein P-PLIN1 (red) but not dNSP2 (lower panel, green). (B) Western blot of immunoprecipitation of PLIN1 and P-PLIN1 from RV-infected cell lysates with anti-perilipin and anti-phospho-perilipin antibodies, showing coprecipitation of vNSP2. (C) Representative images of MA104 cells infected with rRV NSP2 S313D virus at 4 hpi show that vNSP2 colocalized with RV-induced P-PLIN1/LDs before association with NSP5 (examples are indicated with white arrowheads). Scale bar = 5 μ m.

association with lipid droplets (LDs) (2). By 8 h, in a nonrecombinant WT rotavirus infection, we detected vNSP2, but not dNSP2, colocalized with the LD-associated protein phosphorylated perilipin-1 (P-PLIN1) (Fig. 8A). P-PLIN1, in contrast to unphosphorylated PLIN1, is found only on LDs and its presence indicates utilization of the core lipids by β -oxidation and is therefore a convenient and specific marker for determining if viroplasms interact with LDs. Indeed, when either PLIN1 or P-PLIN1 was immunoprecipitated using monoclonal antibodies to the unmodified or phosphorylated forms of perilipin from RV WT virus-infected cell lysates, vNSP2 was coimmunoprecipitated (Fig. 8B). To assess whether vNSP2 or NSP5 was associated with nascent LDs, we probed cells infected with either rRV WT virus or rRV NSP2 S313D virus at 4 hpi. In the rRV WT virus infection, both vNSP2 and NSP5 were found colocalized with P-PLIN1 in discrete puncta (Fig. 8C, upper panel). However, in the rRV NSP2 S313D virus-infected cells, vNSP2 and NSP5 were dispersed throughout the cytoplasm, as expected at that time point (Fig. 8B'), although a few distinct puncta of vNSP2 were observed. Discrete puncta of P-PLIN1 marking LDs were also detected, and upon careful examination, were found to colocalize with vNSP2 D313, but not with NSP5, demonstrating that vNSP2 D313 protein was able to interact with LDs in the absence of NSP5 (Fig. 8C, lower panel). These data suggest that CK1 α -phosphorylated vNSP2 (NSP2 P-S313) can interact with the LD-associated protein P-PLIN1 and that NSP5 is not required for this interaction. This is in agreement with all our data collected thus far, showing that only hyperphos-

phorylated NSP5 interacts with vNSP2 (9) and that, at this time, in the rRV NSP2 S313D mutant virus infection, NSP5 was present only in the 26-kDa hypophosphorylated form (Fig. 3). This also agrees with the recently published data that determined that only hyperphosphorylated NSP5 assembles into viroplasms (18).

DISCUSSION

The general concept of viroplasm formation is a simple one. RV proteins NSP2 and NSP5 are required to interact and assemble into viroplasms. This vision was derived from *in vitro* coexpression assays where the presence of NSP2 and NSP5 was found to be sufficient to form viroplasm-like structures (VLS) (8). This was supported by the fact that when small interfering RNA (siRNA) was used to silence either NSP2 or NSP5 during RV infection, viroplasms failed to form (2, 4). However, with the discovery of two different forms of NSP2, the dispersed form (dNSP2) and the viroplasmic form (vNSP2), a more complex mechanism for viroplasm formation has begun to emerge (9). For example, in cells transiently expressing NSP2 with NSP5 proteins alone, dNSP2 was found sequestered into VLS with NSP5 and not dispersed throughout the cytoplasm as it is during RV infection, indicating that an interaction with other RV proteins is required for the proper localization and, presumably, the function of this form of NSP2 in the cell.

Our discovery that dNSP2 or vNSP2 interact exclusively with either hypophosphorylated or hyperphosphorylated NSP5, respectively, led to the hypothesis of a phosphorylation-dependent mechanism for viroplasm formation (9). This hypothesis was further supported when we determined that NSP2, isolated from RV-infected cells, is phosphorylated on S313 in the CTH and that deletion of the CTH prevented formation of viroplasms (9). We later showed that recombinant NSP2 protein was phosphorylated on S313 by the cellular kinase CK1 α and that a phosphomimetic NSP2 D313 recombinant protein rapidly formed crystals, compared to wild-type NSP2 protein, and was catalyzed by formation of four interoctamer hydrogen bonds between adjacent octamers not formed in the WT protein (17). We know now that CK1 α phosphorylation of NSP2 S313 is required for trafficking vNSP2 to sites of viroplasm formation, where vNSP2, in association with hyperphosphorylated NSP5, initiates viroplasm assembly (17). The work presented here provides further evidence of a phosphorylation-dependent mechanism for viroplasm formation. We showed that phosphorylation of NSP2 S313 is required for efficient replication of the virus as well as for the timely synthesis of hyperphosphorylated NSP5. But how NSP5 first becomes hypophosphorylated to interact with dNSP2 and then becomes hyperphosphorylated in order to interact with vNSP2 and form viroplasms remains unknown.

We have not yet determined what triggers the conversion of dNSP2 into vNSP2. CK1 α phosphorylation of NSP2 at S313 is strongly correlated with the vNSP2 form of NSP2 and appears to be responsible for the trafficking of NSP2 to viroplasm/LD sites of assembly. Our inability to amplify rRV NSP2 S313A virus PCR-positive stock suggests the absolute requirement for NSP2 S313 phosphorylation. It is intriguing to speculate that it is the action of CK1 α on dNSP2 at S313, and the rapid CK1 α -independent transition from dNSP2 to vNSP2 (triggered by an as-yet-unknown mechanism), followed by the interaction of vNSP2 with NSP5 that controls hyperphosphorylation of NSP5 and triggers vNSP2-NSP5 viroplasm assembly. There is strong evidence that CK1 α also plays a direct role in NSP5 phosphorylation. *In vitro*, an NSP5 truncated protein is phosphorylated by CK1 α at serine 67, and a phosphomimetic NSP5 S67D is further phosphorylated independently of CK1 α , suggesting that the initial phosphorylation of S67 by CK1 α is required for NSP5 hyperphosphorylation (23, 24). Indeed, we found that NSP5 remains hypophosphorylated in CK1 α -silenced RV-infected cells and that only the least phosphorylated, 26-kDa form is observed (17). However, transient expression of full-length, wild-type NSP5 did not induce hyperphosphorylated NSP5, demonstrating that CK1 α is in fact not sufficient for NSP5 hyperphosphorylation. It is only when NSP2 is coexpressed with NSP5 that hyperphosphorylated NSP5 isoforms appear, indicating that NSP2 is required for higher-order NSP5 phosphorylation (23). Our previous work supports this finding as we noted that only hypophosphorylated NSP5 was detected in NSP2 siRNA-silenced RV-infected cells, and most notably, that the levels of hypophos-

phorylated NSP5 were also lower than those seen with the CK1 α -silenced RV-infected cells (17). This is striking because CK1 α was present in the NSP2-silenced RV-infected cells and yet NSP5 remained hypophosphorylated, supporting the hypothesis that NSP5 hyperphosphorylation is dependent on the presence of NSP2. In this study, we found that the detection of NSP5 in general was delayed in rRV NSP2 S313D virus-infected cell lysates and that the appearance of NSP5 phosphorylated isoforms corresponding to bands above 26 kDa, particularly the 28-kDa band, was detected far later than in the rRV WT virus-infected cell lysates (Fig. 3), similarly to what was previously observed in CK1 α -silenced and NSP2-silenced cells (17). Since CK1 α was present in cells infected with rRV NSP2 S313D virus, this further implies a requirement for NSP2 in NSP5 phosphorylation. Perhaps during RV infection, it is CK1 α phosphorylation of NSP2, and not of NSP5, that is the key to NSP5 phosphorylation (23).

Recently, a modified reverse genetic method was used to determine the requirement of NSP5 hyperphosphorylation in viroplasm formation and replication (18). A *trans*-complementing cell line stably producing wild-type NSP5 protein was used to characterize various NSP5 phosphorylation-deficient mutant RVs. The study concluded, in agreement with our previous and current work in which we showed that vNSP2 can associate only with hyperphosphorylated NSP5 to form viroplasms, that NSP5 hyperphosphorylation is required for viroplasm assembly. In characterizing the delay in viroplasm assembly using the rRV NSP2 S313D virus, we have shown that viroplasm formation parallels the appearance of hyperphosphorylated NSP5. The rRV NSP2 S313D virus, with a single phosphorylation-specific mutation in NSP2, shows significantly delayed NSP5 hyperphosphorylation, thus supporting the hypothesis that NSP5 hyperphosphorylation is dependent on the presence of NSP2. This is further supported by our data showing that in rRV NSP2 S313D virus-infected cells, at 4 hpi, NSP5 did not associate with LDs, only with vNSP2 D313, because only hypophosphorylated NSP5 was present at that time of the infection. This suggests that the minimal requirement for the association of NSP5 with vNSP2 and LDs is the 28-kDa isoform of NSP5, if not the higher-order hyperphosphorylated isoforms, before viroplasm assembly can proceed.

Our results indicate that the rRV NSP2 S313D virus bypasses a carefully coordinated phosphorylation event that potentially impairs NSP2-dependent phosphorylation of NSP5 and results in delayed formation of viroplasms. Determining the precise sequence of phosphorylation events that lead to viroplasm formation will require careful analysis of the NSP2/NSP5 interaction using combined mutations of both the NSP2 and NSP5 genes by reverse genetics. Furthermore, we expect that these analyses may help us understand the mechanism responsible for the structural organization of NSP2 and NSP5 in the viroplasm and help us to understand what is occurring in the viroplasm core. Recently, superresolution microscopy was used to describe the physical organization of RV proteins within the viroplasmic structure (25). That study determined that NSP5 is located closer to the center of the viroplasm than NSP2, which is detected surrounding NSP5 and more distal from the center. The mechanisms behind the spatial organization of proteins in the viroplasm remain unknown, and data representing the complete crystal structure of NSP5 remain elusive. A structure of full-length NSP2 interacting with peptides of NSP5 was published previously (11) but provides few clues to the overall structure of the viroplasm. Another study of viroplasm formation used transient expression of NSP5 with NSP2, or of NSP5 with VP2, and showed that VP2 can form viroplasm-like structures (VLS) similar to those made by NSP5-NSP2 (26). Interestingly, we previously showed that VP1 and VP2 coprecipitate with both dNSP2 and vNSP2 from RV-infected cell lysates and therefore occur in complex with either hypophosphorylated or hyperphosphorylated NSP5 (9). The contribution of VP1 or VP2 or any of the other RV proteins in assembling viroplasms during RV infection is not known. The goal of our research is to utilize the phosphomimetic NSP2 mutant described here, additional NSP2 and NSP5 phosphorylation mutants, and interacting-domain mutants of the other RV proteins in the viroplasm to understand how and when each interacts to form the viroplasm.

Clearly, the rRV NSP2 S313D phosphomimetic virus is not “hyper”-stimulated to form

viroplasms as originally hypothesized. Instead, the aspartic acid mutation is detrimental to viroplasm formation as evidenced by the slow replication kinetics, the small-plaque phenotype, and the disorganized architecture of the viroplasms. However, the mutation is not lethal. The NSP2 S313D mutation simply slows replication; over time, the virus titers begin to approach those of wild-type virus. It was speculated that the S313D mutation might affect the NTPase or helicase activities of NSP2. However, we know from our previous NSP2 autophosphorylation studies that NTPase activity is unaffected by the mutation (17). And although helicase activity was not assessed, but could be in future studies, it seems unlikely as the effect of a helicase-interfering mutation would be to interfere with replication throughout the replication growth curve and we did not observe this. Rather, these data suggest that we had disturbed the sequential or temporal order of phosphorylation events in viroplasm assembly. Indeed, the NSP2 D313 protein is functional, just impaired. Coinfection with the NSP2 S313D mutant virus interferes with the replication of the WT virus, illustrating the critical role that this residue plays in RV replication and how crucial the timing of phosphorylation is to the virus life cycle. Perhaps the replication of the rRV NSP2 S313D virus is impaired because we have altered the function of dNSP2, which is most often found unphosphorylated at this residue. The ability of the rRV NSP2 S313D virus to recover (e.g., assemble viroplasms, increase NSP5 phosphorylation, and increase titer) suggests that NSP2 S313 phosphorylation by CK1 α is a catalytic event meant to accelerate viroplasm formation and enhance virus replication.

NSP2 is a multifunctional protein and has been implicated in virus replication beyond its role in viroplasm assembly. RV has a segmented, double-stranded RNA (dsRNA) genome consisting of 11 genome segments, and each new virion must contain one example of each segment. How RV packages its genome is not yet known, but NSP2, which nonspecifically binds both single-stranded and double-stranded RNA, may play a role. NSP2 specifically binds the 5' end of single-stranded RNA genome templates and catalyzes the removal of the γ -phosphate required for replication of the second strand (13, 15) and has also been implicated as a chaperone for double-stranded RNA (dsRNA) segment assortment into assembling nascent particles (27). It is entirely possible that additional phosphorylation of specific amino acids within NSP2, which also has autokinase activity in addition to CK1 α phosphorylation (17), governs these functions, turning the protein activity on or off in specific contexts and/or also regulating interactions with other proteins and the genomic RNA.

We used a reverse-engineered RV to shed new light on viroplasm assembly with regard to phosphodependent NSP2 and NSP5 interactions, but a major issue that remains unanswered is that of the exact locations of the "sites" of viroplasm formation. Viroplasms have been shown to associate with LDs (2, 28, 29), and RV infection has been shown to induce LD formation (2, 30). Here, we used a recombinant NSP2 phosphomimetic mutant virus to show that vNSP2 colocalizes with the LD marker protein phospho-PLIN1 without NSP5, suggesting that phosphorylation of NSP2 S313 by CK1 α drives the translocation of NSP2 from the cytoplasm to nascent LDs, where (i) the conversion to vNSP2 occurs, (ii) interaction with hyperphosphorylated NSP5 ensues, and (iii) viroplasm assembly finally begins. Our data suggest that vNSP2 may be the RV factor required for RV infection-induced LD biogenesis. This is an exciting finding because LDs are cellular organelles critical for the replication of many viral and bacterial pathogens, including hepatitis C virus and other viruses of the *Flaviviridae*, as well as for the replication of *Chlamydia* (31–38). Understanding the mechanism of NSP2-mediated viroplasm/LD association and assembly will lead to new insights into this important host-pathogen interaction.

MATERIALS AND METHODS

Cells for transfection and virus infection. A baby hamster kidney (BHK) cell line that stably expresses T7 RNA polymerase (BSR T7/5) (39) was maintained in BHK media, consisting of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Corning), 10% tryptose phosphate broth (Sigma), 1% minimal essential medium (MEM) nonessential amino acids (Thermo Fisher Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), 0.3% sodium bicarbonate (Thermo Fisher Scientific) (7.5%

solution), and 1 mg/ml G418 (Geneticin; Sigma). African green monkey kidney epithelial cells (MA104) were cultured and maintained in DMEM (Sigma) supplemented with 10% FBS (Corning). For maintenance and experiments, all cells were incubated at 37°C and 5% CO₂. Rotavirus strain SA11 4F G3P6[1] was used to infect MA104.

Generation of genetically modified recombinant rotavirus. Recombinant rotavirus SA11 wild-type virus (rRV WT) and recombinant rotavirus SA11 nonstructural protein NSP2 with serine 313 changed to aspartic acid (rRV NSP2 S313D) were generated using the method described previously by Kanai et al. (19). Eleven transcription plasmids carrying RV cDNA for each of the 11 genome segments and 3 additional expression plasmids carrying helper protein cDNA were supplied by Takeshi Kobayashi and acquired through the Addgene plasmid repository (www.addgene.org). The plasmids used were as follows: plasmids 89172 (pT7-NSP5SA11), 89171 (pT7-NSP4SA11), 89170 (pT7-NSP3SA11), 89169 (pT7-NSP2SA11), 89168 (pT7-NSP1SA11), 89167 (pT7-VP7SA11), 89166 (pT7-VP6SA11), 89165 (pT7-VP4SA11), 89164 (pT7-VP3SA11), 89163 (pT7-VP2SA11), 89162 (pT7-VP1SA11), 89161 (pCAG-D12L), 89160 (pCAG-D1R), and 89152 (pCAG-FAST-p10). The plasmid for generating recombinant RV NSP2 S313D was generated by mutating plasmid 89169 (pT7-NSP2SA11) with aspartic acid instead of serine at position 313 (Epoch Life Science). The methods used were as follows. Briefly, all 11 RV plasmids and all 3 helper protein plasmids were transfected (Mirus TransIT-LT1 transfection reagent) into BSR T7/5 cells in a mixture containing DMEM (10% FBS) and incubated for 24 h (37°C, 5% CO₂). The medium was changed at 24 h posttransfection (hpt) to serum-free DMEM (0% FBS). At 48 hpt, MA104 cells were added into the BSR T7/5 transfection plates and cocultured in serum-free media supplemented with 0.5 µg/ml trypsin (Worthington) and incubated for an additional 2 to 4 days. The plates were collected, and the lysates were prepared by three cycles of freeze-thaw. Potential recombinant virus in the coculture lysates was activated with 10 µg/ml trypsin for 30 min and inoculated onto new MA104 cell monolayers for 1 h. After 60 min, the inoculum was removed and replaced with DMEM (0% FBS). Plates were incubated for up to 7 days, depending on visualization of cytopathic effect (CPE), before they were activated in trypsin and passaged again for 7 days (rRV WT virus) or 35 days (rRV NSP2 S313D virus). Following observation of maximal CPE, the virus was passaged repeatedly until sufficient virus was obtained for assays. The virus titer for each recombinant was determined by standard serial dilution plaque assay. Plates were overlaid with neutral red stain at 48 hpi, and plaques were counted at 72 hpi. The titers of virus stock used in this study were as follows: for rRV WT (passage 4), 5.0×10^8 PFU/ml; for rRV NSP2 S313D (passage 5), 3.8×10^8 PFU/ml.

Confirmation of recombinant RV. RV dsRNA from presumptive recombinants was extracted using a QIAamp viral RNA kit (Qiagen) and amplified to cDNA using a SuperScript III One-Step RT-PCR system with Platinum Taq DNA polymerase (Thermo Fisher Scientific). PCR amplification products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced by Genewiz to confirm the NSP2 S313D mutation.

Growth curves. One-step growth curves were performed for both rRV WT and rRV NSP2 S313D viruses on MA104 cell monolayers incubated in DMEM (0% FBS) overnight. Prior to infection, each recombinant virus (MOI = 10) was activated with 10 µg/ml trypsin for 30 min, inoculated onto the MA104 cells, and incubated at 37°C in 5% CO₂ for 60 min. Then the inoculum was removed and replaced with DMEM (0% FBS) and the plates, in triplicate, were allowed to incubate for a total of 2 to 48 h. Growth media and infected cell monolayers were collected at each time point and stored at -80°C until the experiment was completed. The virus was released by freeze/thawing the cells three times, and titers were determined by standard serial dilution plaque assay as described above. Photographs of the plates were taken to assess plaque morphology.

Coinfections with rRV WT and rRV NSP2 S313D viruses. Coinfections were performed for both rRV WT and rRV NSP2 S313D viruses similarly to the growth curves above. Virus was activated with 10 µg/ml trypsin for 30 min. The four conditions used were as follows: (i) rRV WT virus alone (MOI = 10), (ii) rRV NSP2 S313D virus alone (MOI = 10), (iii) rRV WT virus (MOI = 10) coinfecting with rRV NSP2 S313D virus (MOI = 10), and (iv) rRV WT virus (MOI = 10) coinfecting with excess rRV NSP2 S313D (MOI = 20). Each condition was inoculated onto MA104 cells (incubated in DMEM-0% FBS overnight) and incubated at 37°C in 5% CO₂ for 60 min. Then the inoculum was removed and replaced with DMEM (0% FBS) and the plates, in triplicate, were allowed to incubate for 8 h. The virus was released by freeze/thawing the cells three times, and titers were determined by standard serial dilution plaque assay. Neutral red stain was overlaid at 24 hpi, with plaques counted at 48 and 72 hpi. The results reported for the 48-h time point are from replicate wells of two independent experiments. Significance was determined using one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test for comparisons to the WT rRV control (*, $P < 0.05$; **, $P < 0.01$). Results for the 72-h control represent average numbers of plaques from replicate wells of one experiment.

SDS-PAGE separation of RV proteins. rRV WT virus-infected and rRV NSP2 S313D virus-infected cell lysates were prepared in nonreducing or reducing sample buffer as previously described (9). Briefly, to detect NSP5, RV-infected cell lysate was prepared in reducing sample buffer containing β-mercaptoethanol and separated using Criterion TGX precast Midi protein gels (Bio-Rad) (4% to 20%)–1× Tris/glycine/SDS running buffer (Bio-Rad). To detect dNSP2 and vNSP2 proteins, RV-infected cell lysate was prepared in nonreducing sample buffer and separated using Bolt Bis-Tris Plus gels (Thermo Fisher Scientific) (4% to 12%)–1× MES (morpholineethanesulfonic acid) SDS running buffer (Thermo Fisher Scientific) and a Bolt Mini gel tank (Thermo Fisher Scientific). The separated proteins were transferred to nitrocellulose membranes using a Trans-Blot Turbo transfer system (Bio-Rad).

Western blotting. (i) Chemiluminescent Western blotting (Fig. 3) was performed as previously described (9). Mouse monoclonal antibody supernatants corresponding to dNSP2 and vNSP2 (1:1,000)

and polyclonal guinea pig anti-NSP5 (1:1,000) have been previously described (9, 11). Commercially available mouse anti-tubulin (Sigma) (1:1,500) was diluted in a 20% solution of SuperBlock (Tris-buffered saline [TBS]) blocking buffer (Thermo Fisher Scientific). Secondary goat anti-mouse antibody and goat anti-guinea pig antibody conjugated to alkaline phosphatase (Sigma) (1:3,000) were diluted in 20% SuperBlock. Blots were washed once in TBS–0.1% Tween 20, and twice in TBS, before developing was performed with a nitroblue tetrazolium and 5-bromo-4-chloro-3'-indolylphosphate substrate reagent.

(ii) For immunofluorescent Western blotting, membranes were blocked for 1 h at room temperature in 1× casein blocking buffer (Sigma) and immunoblotted using primary antibody to vNSP2 (1:1,000) followed by fluorescent secondary anti-mouse antibodies (LiCor) (1:10,000) diluted in 1× casein blocking buffer. Blots were washed three times with TBS–0.1% Tween 20 and once with TBS prior to visualization using an Odyssey infrared imaging system (LiCor).

Immunoprecipitation and coimmunoprecipitation. A 0.5-ml volume of rotavirus strain SA11 4F G3P6[1] (40) RV-infected or mock-infected cell lysates was transferred to Eppendorf tubes and precleared by incubation with a protein A/G magnetic bead for 1 h at room temperature with rotation. The cleared cell lysates were transferred into new tubes, and 10 µg of anti-perilipin 1 antibody, PLIN1 (catalog no. 4854; Vala Sciences), or anti-phospho-perilipin 1, P-PLIN1 (catalog no. 4855; Vala Sciences), was added. Lysate-antibody mixtures were incubated at 4°C overnight with rotation. Then, a 50-µl volume of protein A/G magnetic beads was added to each mixture followed by incubation at room temperature for 1 h with mixing. Following the incubation, protein A/G beads and interacting antigen-antibody complexes were collected on a magnetic stand. The supernatant was discarded, and the antigen-antibody-bead complex was washed three times in TBST buffer (Tris-buffered saline containing 0.05% Tween 20). Following the washes, 50 µl 2× protein loading buffer (2× Laemmli buffer, without β-mercaptoethanol) was added to the complex followed by boiling at 100°C for 5 min, and the supernatant was used for Western blotting as described above.

Immunofluorescence and confocal microscopy. MA104 cells were grown to confluence on glass coverslips in 24-well culture plates (Costar). In preparation for infection, the MA104 cells were incubated overnight in DMEM (0% FBS). Both rRV WT and rRV NSP2 S313D viruses were activated for 30 min with 10 µg/ml trypsin (Worthington) in DMEM (0% FBS), and the cells were inoculated with the trypsin-activated virus, at a multiplicity of infection (MOI) of 5, for 1 h at 37°C in 5% CO₂. After 60 min, the inoculum was removed and replaced with DMEM (0% FBS) and the coverslips were incubated for the time indicated for each experiment (Fig. 4 and 5; see also Fig. 8) before the medium was removed and the cells were fixed for 30 min at room temperature in 4% paraformaldehyde–phosphate-buffered saline (PBS). The cells were permeabilized in 0.5% Triton X-100 and blocked in 5% bovine serum albumin (BSA). For the primary antibodies, coverslips were incubated as indicated in each figure legend with a 50% mouse anti-dNSP2 or mouse anti-vNSP2 MAb supernatant (9), and guinea pig anti-NSP5 polyclonal antibody (pAb) (1:500), and mouse monoclonal anti-phospho-perilipin antibody, P-PLIN1 (1:500), in a mixture with 2.5% BSA. Coverslips were incubated overnight at 4°C. For the secondary antibodies, the primary antibodies were removed and the coverslips were washed 3 times in PBS. Cells were blocked again for 30 min in 5% BSA prior to addition of (i) goat anti-mouse antibody conjugated to DyLight 488 (Rockland) alone (Fig. 4) or (ii) goat anti-mouse antibody conjugated to DyLight 488 and goat anti-guinea pig antibody conjugated to DyLight 549 (Rockland) (Fig. 5) and were incubated overnight at 4°C. The following day, the cells were washed three times in PBS before NucBlue Fixed Cell ReadyProbes reagent (DAPI [4',6-diamidino-2-phenylindole]; Thermo Fisher Scientific) was added for 5 min. Cells were washed once more in PBS followed by mounting. Confocal imaging was performed as previously described (9). For the experiments described for Fig. 8, mouse MAb supernatant to dNSP2 or vNSP2 and guinea pig polyclonal antibody (pAb) to NSP5 were added to the coverslips, incubated, and removed as indicated above. To detect MAb mouse anti-P-PLIN1, 1 µg of antibody was directly end labeled using a Zenon Alexa Fluor 568 or 647 mouse IgG1 labeling kit (Thermo Fisher) for each coverslip. End-labeled anti-P-PLIN1 was incubated on the coverslips for 1 h at room temperature and removed, and the coverslips were washed 3 times with PBS followed by fixing the coverslips once more with 5% PFA for 10 min as indicated by the manufacturer's instructions. The cells were then washed three times in PBS before NucBlue Fixed Cell ReadyProbes reagent was added for 5 min. Cells were washed once more in PBS before mounting and confocal imaging were performed.

All commercial primary and secondary antibodies were tested for rotavirus antibodies by Western blotting using mock-infected and rotavirus-infected cell lysates and purified rotavirus and/or confocal microscopy examination of mock and rotavirus-infected cells (data not shown). Any antibody that contained antibodies against rotavirus was not used.

Image analysis. Image analysis comparing vNSP2 and viroplasms formed by the rRV WT and rRV NSP2 S313D viruses was performed using the open source software CellProfiler 2.2 (cellprofiler.org). The pipeline was designed to first identify nuclei from the DAPI signal and then to expand the nuclear mask to approximate the cytoplasmic region; then, viroplasms were also segmented and related to the cytoplasmic mask, allowing determination of area measurements and viroplasm counts/cell. The available texture features were calculated at three angles and a kernel of 10 from the viroplasm channel in the cytoplasm mask. Graphs were made in GraphPad Prism, and the heat map was built in Orange (41).

Transmission electron microscopy. rRV WT virus-infected and rRV NSP2 S313D virus-infected MA104 cells were trypsinized and fixed in 2% paraformaldehyde–3% phosphate-buffered glutaraldehyde at the indicated time points. Samples were stabilized in a pellet using 5% bovine serum albumin and overlaid with buffered glutaraldehyde. Postfixation was carried out in 1% buffered osmium tetroxide and dehydrated through graded ethanol, and cells were embedded in Polybed 812. Thin sections were stained with uranyl acetate and lead citrate. Grids were viewed and photographed using a FEI Tecnai

Spirit transmission electron microscope equipped with a 4K Eagle digital camera. Only the viroplasmic structures associated with virus particles were counted by visual examination of 41 image fields from rRV WT virus-infected cells and of 45 image fields from rRV NSP2 S313D virus-infected cells.

Data analysis. All of the statistical analyses were performed using ANOVA in GraphPad Prism version 6.0 for Windows (GraphPad Software), and *P* values of <0.05 were considered to be statistically significant.

ACKNOWLEDGMENTS

This work was primarily supported by National Institutes of Health (NIH) grant R01 AI080656. This project was also supported by the Advanced Technology Core Laboratories (Baylor College of Medicine), specifically, by the Integrated Microscopy Core at Baylor College of Medicine, with funding from NIH (DK56338, CA125123, and ES030285) and CPRIT (RP150578 and RP170719), the Dan L. Duncan Comprehensive Cancer Center, and the John S. Dunn Gulf Coast Consortium for Chemical Genomics.

We thank the Texas Medical Center Digestive Diseases Consortium core facilities, particularly Jim Barrish at the Electron Microscopy Laboratory at Texas Children's Hospital, Houston, TX.

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