Actin-Dependent Nonlytic Rotavirus Exit and Infectious Virus Morphogenetic Pathway in Nonpolarized Cells

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ABSTRACT During the late stages of rotavirus morphogenesis, the surface proteins VP4 and VP7 are assembled onto the previously structured double-layered virus particles to yield a triple-layered, mature infectious virus. The current model for the assembly of the outer capsid is that it occurs within the lumen of the endoplasmic reticulum. However, it has been shown that VP4 and infectious virus associate with lipid rafts, suggesting that the final assembly of the rotavirus spike protein VP4 involves a post-endoplasmic reticulum event. In this work, we found that the actin inhibitor jasplakinolide blocks the cell egress of rotavirus from nonpolarized MA104 cells at early times of infection, when there is still no evidence of cell lysis. These findings contrast with the traditional assumption that rotavirus is released from nonpolarized cells by a nonspecific mechanism when the cell integrity is lost. Inspection of the virus present in the extracellular medium by use of density flotation gradients revealed that a fraction of the released virus is associated with low-density membranous structures. Furthermore, the intracellular localization of VP4, its interaction with lipid rafts, and its targeting to the cell surface were shown to be prevented by jasplakinolide, implying a role for actin in these processes. Finally, the VP4 present at the plasma membrane was shown to be incorporated into the extracellular infectious virus, suggesting the existence of a novel pathway for the assembly of the rotavirus spike protein.

IMPORTANCE Rotavirus is a major etiological agent of infantile acute severe diarrhea. It is a nonenveloped virus formed by three concentric layers of protein. The early stages of rotavirus replication, including cell attachment and entry, synthesis and translation of viral mRNAs, replication of the genomic double-stranded RNA (dsRNA), and the assembly of double-layered viral particles, have been studied widely. However, the mechanisms involved in the later stages of infection, i.e., viral particle maturation and cell exit, are less well characterized. It has been assumed historically that rotavirus exits nonpolarized cells following cell lysis. In this work, we show that the virus exits cells by a nonlytic, actin-dependent mechanism, and most importantly, we describe that VP4, the spike protein of the virus, is present on the cell surface and is incorporated into mature, infectious virus, indicating a novel pathway for the assembly of this protein.

KEYWORDS cell exit, morphogenesis, rotavirus, virus assembly

Rotaviruses are naked double-stranded RNA (dsRNA) viruses that represent important etiological agents of gastroenteritis in infants and young children. These viruses are formed by three concentric layers of protein that surround the viral genome, which is composed of 11 segments of dsRNA. The outer layer is constituted by two proteins: VP7, which forms the smooth surface of the virus, and VP4, which makes the spikes of the virus particle and mediates virus attachment and penetration of the virus particle into the cell (1). It has also been described that VP4 interacts with different

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proteins and cellular structures during virus replication; at early times postinfection, it has been found on the cell surface and associated with microtubules (2), cellular lipid rafts (3), the small GTPase Rab5, and the prenylated Rab acceptor PRA1 (4). Recently, Li et al. published the VP4 interactome, and interestingly, they found that this protein interacts with several cytoskeleton-associated proteins (5).

Most of our knowledge about the life cycle of rotavirus has been obtained from studies carried out with MA104 cells, derived from rhesus monkey kidney epithelium. The early stages of rotavirus replication, including cell attachment and entry, synthesis and translation of viral mRNAs, and formation of viroplasms, electron-dense cytoplasmic structures where the replication of dsRNA and assembly of double-layer viral particles occur, are widely documented (6–11). However, the mechanisms involved in the later stages of infection, i.e., viral particle maturation and cell exit, are less well characterized. Once the double-layer particles (DLPs) are assembled in the viroplasms, VP7, an endoplasmic reticulum (ER) membrane-resident glycoprotein, and VP4, a cytosolic protein, have to be incorporated into DLPs to form mature, infectious triple-layer particles (TLPs). DLPs bud from the viroplasm into the lumen of the ER through interaction with NSP4, a second ER membrane-resident nonstructural viral protein, acquiring during this process a transient membrane envelope that is subsequently lost during the final assembly of mature, infectious TLPs (1). Since knocking down the expression of VP7 by RNA interference (RNAi) leads to an accumulation of enveloped DLPs in the lumen of the ER (12), it has been suggested that VP7 is necessary for removal of the lipid envelope concomitant with its assembly onto DLPs in this cellular compartment. However, where and how VP4 is incorporated into TLPs are unclear. The most accepted model is that this protein is located in the space between the viroplasm and the ER membrane, forming a heterotrimer with NSP4 and VP7, and during the budding process is internalized with VP7 into the lumen of the ER, where it assembles when the lipid envelope is removed (1). It has also been described, however, that VP4 is assembled at a post-ER event, in association with raft microdomains, during the egress of the virus from infected cells (13, 14). In fact, it has been suggested that rotavirus uses lipid rafts as final assembly platforms, since VP4 associates with these detergent-resistant membranes as early as 3 h postinfection (hpi) and the infectious virus can be recovered from these lipid microdomains (3). Interestingly, studies performed on polarized Caco-2 cells have shown that rotavirus is released from the apical surface of the cells through a nonconventional, nonlytic vesicular transport method (15). On the other hand, it has historically been assumed that rotavirus exits nonpolarized cells by a nonspecific lytic process.

The actin cytoskeleton is a highly dynamic network that maintains the cell shape and plays a major role in surface remodeling, transmembrane signaling, endocytosis, and secretion (16). Viruses can interact with actin, rearranging it to facilitate their infection process (17). During rotavirus replication, changes in the organization of the cytoskeleton occur (18), although the role of this reorganization is unclear.

In the present study, we show that at early times of infection rotavirus exits nonpolarized MA104 cells by a nonlytic mechanism that is actin dependent and that the virus particles present in the extracellular medium are associated with low-density membranous structures. Also, we found that in MA104 cells a fraction of VP4 localizes to the surface of the plasma membrane and that this subset of the spike protein is incorporated into mature, infectious particles, indicating a novel pathway for virus assembly.

RESULTS

Release of rotavirus from nonpolarized MA104 cells is decreased in the presence of jasplakinolide. Previous reports have described the effects of drugs that affect the actin cytoskeleton during the early steps of rotavirus replication (19). In this work, we evaluated the effects of different actin cytoskeleton inhibitors on later stages of the rotavirus life cycle. The following three drugs with various modes of action were tested: latrunculin, cytochalasin, and jasplakinolide (JAS). For this purpose, nonpolarized

FIG 1 Release of rotavirus from MA104 cells decreases in the presence of JAS. MA104 cells were infected with RRV (MOI = 3). At 4 hpi, JAS (1 μ M), latrunculin (1 μ M), cytochalasin (10 mM), or DMSO (in the case of control cells) was added and kept in the medium; at 14 hpi, the amount of total infectious virus (virus present in the medium and cell-associated virus) (A) or virus present only in the medium (B) was quantitated as described in Materials and Methods. (C) Different concentrations of JAS or DMSO (in the case of control cells) were added after cell infection, and the drugs were kept in the medium; at 14 hpi, the virus present in the medium was quantitated. (D) JAS (1 μ M) was added to infected cells at different times postinfection (0, 4, 8, and 10 h), and at 14 hpi, the amount of virus in the cell medium was quantitated as described above. Data are expressed as percentages of infectious virus present in the medium or of total virus compared to that in control cells treated with DMSO, which was taken as 100%. The arithmetic means and standard deviations for four independent experiments performed in triplicate are shown. **, $P < 0.01$; ***, $P < 0.001$.

MA104 cells were infected with the rhesus rotavirus strain RRV at a multiplicity of infection (MOI) of 3, and at 4 hpi, latrunculin (1 μ M), cytochalasin (10 mM), or JAS (1 μ M) was added and kept in the cell medium until cell harvest. In these assays, the highest concentrations of the different drugs that did not affect cell viability, as verified by lactate dehydrogenase (LDH) release and resazurin reduction assays (data not shown), were used. At 14 hpi, the titers of total virus (cell-associated virus plus that present in the extracellular medium) and virus released from cells (virus present in the extracellular medium) were determined by an immunoperoxidase assay as reported previously (20). None of the drugs affected the total yield of infectious virus (Fig. 1A); however, treatment with JAS decreased the amount of rotavirus present in the extracellular medium by about 60% (Fig. 1B). JAS decreased the egress of the virus in a dose-dependent manner (Fig. 1C) and was more effective when added at 4 hpi (Fig. 1D).

The functionality of all three inhibitors was evaluated by analyzing the changes of the intracellular actin distribution pattern as detected by immunofluorescence microscopy (shown in Fig. 2A and B for JAS). The immunofluorescence pattern in control cells showed the characteristic cytoplasmic filaments and cortical actin that define the cellular border, while JAS-treated cells showed a lack of actin bundles in the cytoplasm and the formation of aggregates of actin. The effect of JAS on cells was also evaluated

FIG 2 Jaspilakinolide affects the actin cytoskeleton structure. MA104 cells were left untreated (DMSO) (A) or treated with JAS ($1\ \mu\text{M}$) (B) for 14 h at 37°C , fixed, immunostained, and analyzed by immunofluorescence assay. Actin filaments were stained with phalloidin coupled to Alexa 448 (green), and nuclei were stained with DAPI (blue). (C and D) Electron micrographs of MA104 cells that were left untreated (C) or treated with $0.5\ \mu\text{M}$ JAS (D) for 4 h at 37°C . Cells were fixed and embedded as described in Materials and Methods. ER, endoplasmic reticulum; Gg, Golgi apparatus; m, mitochondria; Nu, nucleus; AF, actin filaments, MT, microtubules. The arrows indicate the ER membranes.

by transmission electron microscopy (TEM). In untreated control cells, mitochondria, the ER, the Golgi apparatus, and microtubule bundles could clearly be observed (Fig. 2C). In contrast, in cells treated with JAS, an agglomeration of mitochondria and a large number of autophagosomes were found, the ER and the Golgi apparatus appeared to be disaggregated, and the microtubule bundles could no longer be distinguished (Fig. 2D).

Kinetics of rotavirus cell release. The observation that treatment with JAS decreased the amount of virus present in the extracellular medium suggested that at least some of the rotavirus particles might exit MA104 cells by an actin-dependent mechanism. To further characterize this observation, a time course study of total and released

FIG 3 JAS affects the kinetics of rotavirus cell release. MA104 cells were infected with RRV (MOI = 3), either JAS (1 μ M) or DMSO (control cells) was added at 4 hpi, and total virus (A) or the virus present in the extracellular medium (B) was quantitated at the indicated times as described in Materials and Methods. Data are expressed in FFU per milliliter. (C) Percentages of infectious virus present in the medium for JAS-treated cells compared to that for control cells treated with DMSO, which was taken as 100%. Infected or mock-infected cells, treated or not with JAS, were assayed for resazurin reduction (D) and LDH release (E) at the indicated times postinfection. The arithmetic means $\pm$ standard deviations for four independent experiments performed in triplicate are shown. **, $P < 0.001$.

virus from JAS-treated cells was performed. MA104 cells were infected with RRV, JAS (1 μ M) was added at 4 hpi, and the amount of infectious virus present in the extracellular medium and the total amount of virus produced (cell associated and present in the extracellular medium) were determined at the indicated times. It was found that the amount of total viral progeny produced peaked at 12 hpi, and the presence of JAS did not affect its production at any of the times tested (Fig. 3A). In contrast, a difference in the amount of released virus was observed for JAS-treated cells compared to untreated cells (Fig. 3B). In control, untreated cells, the virus was initially detected in the cell medium at about 9 hpi, reaching its highest concentration at 14 hpi, while in the presence of JAS a delay in release of virus of about 2 h was observed. In both control and JAS-treated cells, the virus levels in the cell medium reached similar concentrations by 16 hpi and afterward (Fig. 3B). From 9 to 14 hpi, the drug inhibited the cell release of the virus by about 60% (Fig. 3C). These findings confirm that JAS affects the release of the virus but not the formation of total infectious particles. Also, the observation that an actin cytoskeleton inhibitor blocks virus release strongly suggests that at least a fraction of the virus can exit nonpolarized cells by a nonlytic mechanism.

The release of LDH and the reduction of resazurin were assessed at different times postinfection to evaluate the integrity of the cells. As judged from these two indicators, there was no significant change in cell viability in cells treated with JAS (Fig. 3D and E). A decrease in cell viability was initially observed at 12 hpi for rotavirus-infected cells, regardless of whether they had been treated with the drug or not. These observations suggest that the virus begins to exit cells at 9 to 10 hpi, when there is neither a loss of membrane integrity nor inhibition of metabolic activity. Importantly, despite the fact that at 14 hpi the cells showed evidence of a loss of membrane integrity, JAS still affected the release of the virus at this time (Fig. 3C), supporting a role of actin in virus release even under conditions where cell viability starts to be compromised.

A fraction of released virus is associated with low-density membranous structures. Previous reports have shown that nonenveloped viruses can be released from infected cells by a nonlytic process, generally associated with extracellular vesicles (21–24). To evaluate if this could be the case for rotavirus, the virus particles present in the cell culture medium of RRV-infected MA104 cells were analyzed by use of density gradients. Confluent monolayers of MA104 cells were infected with RRV, and at 10 hpi the cell medium was collected and centrifuged at low speed to remove cells that had

FIG 4 A fraction of released infectious virus associates with low-density membranous structures. MA104 cells were infected with RRV (at an MOI of 3), and at 10 hpi the extracellular medium was collected. Viral particles present in the medium of untreated control cells (A), JAS-treated cells (C), or cells not treated with the drug but treated with 1% Triton X-100 (TX-100) for 30 min at room temperature (E) were fractionated by use of iodixanol gradients as described in Materials and Methods. Twelve 400- μ l fractions were collected from the top of the tube, and the proteins present in each fraction were separated by SDS-PAGE (10% gel) and analyzed by Western blotting using anti-TLP antibodies. The viral titer of each fraction was quantitated as described in Materials and Methods, and titers are shown in panels B, D, and F. (G) Fractions from the gradient shown in panel A were left untreated or treated with 1% Triton X-100 for 30 min at room temperature and subsequently incubated or not with trypsin (10 μ g/ml) for 30 min at 37°C, and the viral titer of each fraction was quantitated as described above. The arithmetic means $\pm$ standard deviations for three independent experiments are shown.

detached during the incubation period. The virus was then centrifuged in a discontinuous iodixanol flotation gradient as described in Materials and Methods, and twelve 400- μ l fractions were subsequently collected and analyzed by Western blotting with a hyperimmune serum against rotavirus (anti-TLPs). Viral proteins were detected in all fractions of the gradient, indicating that at least some of the released virus particles were associated with low-density membranous structures, most prominently in fractions 2 to 6 (Fig. 4A). In parallel, the presence of infectious virus in the gradient fractions was determined. Besides the infectious viral particles that remained in the bottom fractions of the tube, significant amounts of infectious virus were found in fractions 3 to 6 (Fig. 4B). It is interesting that the treatment of infected cells with JAS did not affect the flotation pattern of infectious extracellular viral particles (Fig. 4C and D), indicating that the drug does not modify the interaction of the virus with membranes but rather its actin-dependent egress. As a control to confirm the association of the virus with

FIG 5 Cellular distribution of VP4 is affected by JAS. MA104 cells infected with RRV were treated or not with 1 μ M JAS. The drug was added either at 0 hpi, in the case of cells that were fixed at 4 and 6 hpi, or at 4 hpi, for cells that were fixed at 10 hpi. VP4 was stained with MAb HS2 and goat anti-mouse IgG coupled to Alexa 448 (green). NSP2 was stained with a rabbit polyclonal antibody and a goat anti-rabbit antibody coupled to Alexa 568 (red). Nuclei, shown in blue, were stained with DAPI.

membranous structures, the virus in the cell medium of untreated cells was treated with Triton X-100 before density gradient centrifugation. Treatment with detergent abolished the presence of the virus in the low-density fractions (Fig. 4E), corroborating the finding that the virus present in fractions 1 to 6 was associated with cellular membranes. Virus infectivity also essentially disappeared from these fractions (Fig. 4F). Additionally, the infectivity of the virus in the low-density fractions of the gradient increased about 7- to 10-fold when cells were treated with Triton X-100 before trypsin treatment, and this increase in infectivity was also observed even in the samples that were not treated with trypsin (Fig. 4G). Taken together, these results suggest that rotavirus particles are associated with cell membranes during their exit from infected MA104 cells and that these membranes partially hide viruses from access by trypsin.

Intracellular distribution of VP4 is affected by JAS. VP4 is synthesized as a cytosolic protein, and it has been reported to localize in the space between viroplasm and the ER membrane and to associate with lipid rafts (3, 13, 25). To evaluate if JAS affects the intracellular localization of VP4, we evaluated its distribution by immunofluorescence assay. For this purpose, untreated or JAS-treated (with JAS added at the times indicated in the legend to Fig. 5) MA104 cells were infected with RRV, and at 4, 6, and 10 hpi the cells were fixed, incubated with a monoclonal antibody (MAb) to VP5 (clone HS2) (26), and analyzed. In untreated control cells, the VP4 staining pattern showed that the protein was localized around or partially surrounding viroplasms as well as diffusely distributed in the cytoplasm at all times analyzed (Fig. 5, control panels). However, when infected cells were treated with JAS, the staining of VP4 was restricted to the surroundings of viroplasms in the perinuclear region. It is interesting that in JAS-treated cells at 10 hpi, the viroplasms also seemed to be restricted to a region around the nucleus compared to those in control, untreated cells (Fig. 5, JAS panels). Similar results were obtained when MAb 2G4, which recognizes an epitope different from that for HS2 (27), was used (data not shown). These observations suggest that the intracellular distribution of VP4 in infected MA104 cells is influenced by an actin mechanism inhibited by JAS.

Functional actin filaments are required for association of viral particles with lipid rafts. As mentioned above, it has been reported that lipid rafts are necessary for viral transport to the cell surface and release of viruses from polarized Caco-2 cells (13) and that infectious viruses interact with lipid rafts in MA104 cells (28). Since the

FIG 6 JAS disrupts the association between lipid rafts and viral particles. Infected MA104 cells were treated or not with JAS (1 μ M). At 10 hpi, the cells were lysed and rafts were purified as described in Materials and Methods. (A) Eleven fractions were collected from each iodixanol gradient and analyzed by Western blotting using the anti-TLP antibody. As a raft marker, the presence of ganglioside GM1 was detected by dot blotting using the cholera toxin B subunit conjugated to horseradish peroxidase. (B) Infectivity assay. The titer of each fraction was determined on MA104 cells as described in Materials and Methods. Data are expressed as percentages of infectious virus present in the fractions from JAS-treated infected cells compared to that for infectious virus in each control fraction, which was taken as 100%. The arithmetic means and standard deviations for three independent experiments are shown. ***, $P < 0.001$.

association between rafts and viral particles seems to be important for rotavirus cell egress, we hypothesized that the actin cytoskeleton may be involved in this association. To test this possibility, the interaction between rotavirus and lipid rafts was analyzed in cells treated with JAS. For this purpose, MA104 cells were infected with RRV, and at 4 hpi, the culture medium was replaced by Eagle's minimum essential medium (MEM) containing 1 μ M JAS. The cells were incubated until 10 hpi and then collected, and the lipid rafts were isolated by density centrifugation as described in Materials and Methods. After gradient fractionation, the collected samples were analyzed by Western blotting using anti-TLP antibodies; ganglioside GM1 (29) was used as a marker for rafts. For control cells, viral proteins were detected in fractions 4 to 11, while GM1 was enriched in fractions 4 to 7, corresponding to detergent-resistant membranes floating in the density gradient (Fig. 6A, left panel), consistent with previous reports (28). Interestingly, the association of rotaviral proteins and infectious viral particles with rafts in the lower-density fractions, particularly in fractions 4 to 7, was decreased by about 60 to 70% in the presence of JAS (Fig. 6A, right panel; quantified for three different experiments in Fig. 6B). These observations suggest that a functional actin cytoskeleton facilitates the interaction of infectious virus with lipid rafts.

Targeting of VP4 to the cell surface is affected by JAS. Despite its being synthesized as a cytosolic protein, a significant amount of VP4 has been found on the cell surface of MA104 cells (2). To test the effect of JAS on this localization, cells were infected with RRV and treated with the drug for 6 h before labeling of the cell surface proteins with a membrane-impermeant biotinylation reagent (sulfo-NHS-LC-biotin) for 30 min, at 6, 8, and 10 hpi. Cells were lysed at these times, and the biotinylated proteins were purified by use of streptavidin-coupled magnetic beads. The streptavidin-purified and total cell proteins were then analyzed by Western blotting using polyclonal anti-TLP serum. As can be seen in Fig. 7A, the viral proteins (VP4, VP6, and VP7) from JAS-treated and untreated cells were detected at similar levels in the total protein samples; in contrast,

FIG 7 Transport of VP4 to the plasma membrane is affected by JAS treatment. MA104 cells were infected with RRV (at an MOI of 3) and left untreated or treated with JAS (1 μ M) for 6 h before being biotinylated. At the indicated times postinfection, the cells were biotinylated as described in Materials and Methods. The biotinylated proteins were precipitated by use of streptavidin (Strp) magnetic beads and analyzed by Western blotting using the anti-TLP antibody. The loading control was developed with a streptavidin-peroxidase conjugate. (A) Representative Western blot of proteins harvested at 6 hpi. A biotinylated membrane protein was used as a loading control. (B) Quantification of the biotinylated VP4 present at the plasma membrane by densitometry analysis as described in Materials and Methods. The amount of VP4 on the surfaces of control untreated cells at each time postinfection was taken as 100%. The arithmetic means and standard deviations for three independent experiments are shown. **, $P < 0.01$; ***, $P < 0.001$.

only VP4 was detected in the streptavidin-purified proteins, confirming the previous observations that VP4 localizes to the plasma membrane, that the biotinylation reagent does not penetrate the cell membrane, and that biotinylation is restricted to proteins exposed on the cell surface (Fig. 7A). Densitometric analysis of the streptavidin-purified versus total VP4 protein showed that about 35% of the synthesized VP4 protein was accessible to biotinylation on the cell surface for control, untreated cells (data not shown). On the other hand, a significant decrease of biotinylated VP4 was observed for cells infected in the presence of JAS compared to that for control cells, and this decrement, of about 50 to 60%, was observed at all three postinfection times evaluated (Fig. 7B).

VP4 present at the plasma membrane is incorporated into viral particles. Since a significant fraction of VP4 seems to localize at the cell surface during the infection process, we wondered whether this protein was assembled into virions. To test this possibility, cell surface VP4 was mock labeled or labeled with biotin (for 30 min at 6 hpi, as described above) in RRV-infected cells, and after thorough washing to remove the biotinylation reagent, the cells were incubated until 12 hpi to allow the protein to be incorporated into viral particles. At this time, the cells were lysed, and the TLPs were purified by banding twice in consecutive CsCl density gradients. The TLPs from biotinylated and nonbiotinylated cells were purified by use of streptavidin-coupled magnetic beads, and the viral proteins were analyzed by Western blotting using the anti-TLP hyperimmune serum. When total TLPs were analyzed, the viral proteins were detected in similar amounts for both biotin-labeled and unlabeled viruses. In contrast, for the streptavidin-purified TLPs, the viral proteins were detected only in the virus derived from biotin-labeled cells (Fig. 8A). To confirm the incorporation of biotinylated cell surface VP4 into mature TLPs, total virus particles from biotinylated and nonbiotinylated cells were purified as described above; these viruses were then incubated with a streptavidin-gold conjugate and examined by TEM. In 12.5% (27/217 particles) of the viral particles from biotinylated cells, one or two gold beads bound to the viral surface were observed, while in the virus purified from control, nonbiotinylated cells, only 2.6% (3/115 particles) of the viral particles were labeled with one gold particle (Fig. 8B). Since total virus (intracellular plus extracellular) was extracted from cells and used for this characterization, it is not surprising that only one-eighth of the particles were labeled with gold beads, since they most likely represent the viruses present in the extracellular

FIG 8 VP4 present at the plasma membrane is incorporated into viral particles. MA104 cells were infected with RRV (at an MOI of 3 FFU/cell), and at 6 hpi the cells were biotinylated with the impermeant reagent sulfo-NHS-LC-biotin for 30 min. The cells were then washed and incubated until 12 hpi. At this time, TLPs were purified twice by use of CsCl isopycnic gradients. (A) TLPs obtained from biotinylated (Biot) and nonbiotinylated (Ctrl) cells were purified by use of streptavidin (Strp) magnetic beads and analyzed by Western blotting using the anti-TLP antibody. (B) Control TLPs (1) and biotinylated TLPs (2) were stained with streptavidin-gold and analyzed by electron microscopy as described in Materials and Methods.

medium, which represent about 4% of the total virus at 12 hpi (Fig. 3). Altogether these results indicate that the VP4 protein exposed on the cell membrane is incorporated into virions.

Electron microscopy imaging of RRV-infected MA104 cells treated with jasplakinolide. To further characterize the effect of JAS on rotavirus replication, RRV-infected MA104 cells in the absence or presence of the drug were analyzed by TEM at 10 hpi. For this purpose, MA104 cells were infected with 25 viroplasm-forming units (VFU) of RRV per cell or mock infected; at 6 hpi, the medium was replaced by MEM containing 0.5 μ M JAS in 2% dimethyl sulfoxide (DMSO) or 2% DMSO alone, and 4 h later, the cells were fixed and processed for EM. The same structural alterations observed in noninfected cells in the presence of JAS (Fig. 2) were observed in RRV-infected cells in the presence of the drug. However, consistent with the observation that JAS does not decrease the production of total TLPs, it did not seem to affect the assembly and budding of DLPs into the ER, since viral particles within the lumen of the ER adjacent to viroplasms, a distinctive characteristic of rotavirus infection, were observed in both the absence (Fig. 9A) and presence (Fig. 9B) of JAS. On the other hand, it was interesting that at this time postinfection (10 hpi) the presence of enveloped virions within smooth vesicles and ER vesicles (as judged by the presence of ribosomes on the vesicular membrane) was localized far from viroplasms and close to the plasma membrane (Fig. 9C and D). These vesicles were found in different places in the cells, indicating that their presence was not a fortuitous event and suggesting that viruses can be transported to the cell surface within vesicles.

Requirement of a functional actin cytoskeleton for release of different rotavirus strains from MA104 cells is conserved. We evaluated if the function of actin filaments is required for the release of different rotavirus strains from nonpolarized MA104 cells. For this purpose, cell monolayers were infected with the simian RRV, bovine UK, or human Wa rotavirus strain (all at an MOI of 3), and JAS was added or not to the cell medium at 4 hpi. At 12 hpi, the virus in the supernatant was collected and its infectious titer determined. As shown in Fig. 10, incubation of cells with JAS inhibited the release of all tested rotaviruses, to similar extents, suggesting that a nonlytic exit of the virus, dependent on the actin cytoskeleton, is conserved among different rotavirus strains.

FIG 9 Electron microscopy analysis of MA104-infected cells in the presence of JAS. MA104 cells were infected with RRV at an MOI of 25 VFU per cell, and at 6 hpi the cells were left untreated (DMSO) (A) or treated with 0.5 μ M JAS (B). At 10 hpi, the cells were fixed and embedded as described in Materials and Methods. (C and D) RRV-infected MA104 cells, as described above, in the absence (C) or presence (D) of 0.5 μ M JAS. The dashed boxes show viral particles within vesicles close to the plasma membrane and far from viroplasm; in the right panels, higher-magnification views of the dashed boxes from the left panels are shown. ER, endoplasmic reticulum; m, mitochondria; Nu, nucleus; V, viroplasm. Also, viral particles in smooth vesicles (open arrows) and ER vesicles (black arrowheads) are shown.

DISCUSSION

It has been found that rotavirus is released almost exclusively from the apical surface of polarized cells before any sign of cell lysis (15). On the other hand, it has traditionally been thought that rotaviruses are released from nonpolarized cells through a lytic process when the integrity of the cells is compromised; nevertheless, the studies supporting these observations were performed at late times of virus infection, when the cells had an extensive cytopathic effect (30–32). In the present work, we found that the release of rotavirus particles from nonpolarized MA104 cells begins when there is no evidence of cell lysis, through a mechanism that depends on a functional actin

FIG 10 Jasplakinolide affects the release of different rotavirus strains. MA104 cells were infected with the indicated rotavirus strains (at an MOI of 3 FFU/ml). At 12 hpi, the extracellular medium was collected and the released virus was quantified as described in Materials and Methods. Data are expressed as percentages of the amount of infectious virus present in the medium of control cells treated with DMSO, which was taken as 100%. The arithmetic means and standard deviations for three independent experiments performed in triplicate are shown.

cytoskeleton. JAS, which induces actin polymerization and stabilizes the actin filaments (33), was found to inhibit the cell release of rotavirus by about 60%. Similar inhibitory effects of JAS have been reported for the cell exit of respiratory syncytial virus (34), measles virus (35), and dengue virus (36). Also, Gardet et al. showed that inhibition of actin treadmilling by JAS resulted in a loss of the preferential apical release of rotavirus from Caco-2 cells, suggesting a major role for actin in virus release from polarized cells (37). In our hands, JAS treatment of cells also inhibited the release of RRV from nonpolarized Caco-2 cells by about 60% (data not shown), suggesting that a nonlytic, actin-dependent mechanism is conserved between different cell lines. Moreover, this is also true for different rotavirus strains, since the cell exit of the three rotaviruses tested, of simian (RRV), bovine (UK), and human (Wa) origins, was inhibited by JAS. It is interesting that JAS, but not cytochalasin or latrunculin, affected virus release, especially given that all three inhibitors disrupt actin. However, cytochalasins B and D as well as latrunculin A have been reported to disassemble actin stress fibers, while JAS, although it disaggregates actin filaments, does not disassemble stress fibers (38), which may be necessary for rotavirus egress.

It is noteworthy that even though the amount of virus present in the supernatant represents only about 4% of the total viral progeny produced at early times of infection, the percentage of virus present in the extracellular medium reaches a maximum of 10% of the total virus produced at 36 hpi, when there is evidence of abundant cell lysis (data not shown). Consistent with these results, it has been reported that a large proportion of rotavirus remains associated with MA104 cell structures at late times of infection (32). These data suggest that although a fraction of the extracellular virus may be released as a consequence of cell death at later times of infection, a significant proportion of the total extracellular infectious virions exit the cell through a nonlytic mechanism. In contrast to these observations, it seems that the virus can be released more efficiently from polarized cells than from nonpolarized cells, at least in cell culture, since it was reported for rotavirus-infected polarized Caco-2 cultures that the virus in the extracellular apical medium reaches about 98% of the total viral progeny at 24 hpi (15). It is interesting that despite the different percentages of extracellular virus found with MA104 and polarized Caco-2 cells, concerning the total viral progeny produced, JAS treatment reduced the release of rotavirus to similar extents in both cell lines (this work and reference 15).

How is a naked virus released from cells without breaching the plasma membrane barrier? In recent years, single-stranded positive-sense naked viruses have been associated with nonlytic spread; although the detailed mechanism of virus egress is not known, several strategies have been suggested, such as the creation of new vesicular compartments or exosomes that allow the exit of cytoplasm and its contents without violating the integrity of the cell (39). In this work, we observed what appeared to be immature, enveloped virions enclosed in vesicles near the plasma membrane, similar to what has been reported for polarized Caco-2 cells, although in the latter case the

vesicle-enclosed virus particles appeared to be mature, suggesting that newly synthesized viruses are transported to the plasma membrane through a vesicular process (15). It has also been suggested that vesicular transport mediated by rafts is a plausible pathway for rotavirus to reach the cell surface (13). Furthermore, it has been postulated that VP4 is responsible for the association of rotavirus particles with rafts, since this viral protein was detected in rafts at early infection times (3); nevertheless, where and how VP4 interacts with lipid rafts are still unclear. Our results show that the intracellular distribution of VP4 in MA104 cells is actin dependent and that the association of rotavirus with rafts is disrupted by JAS. Thus, it can be hypothesized that the interaction of VP4 with actin facilitates its association with lipid rafts. In support of this observation, a recent characterization of the VP4 interactome revealed that this viral protein has a strong interaction with actin and other components of the actin cytoskeleton (5). On the other hand, it is worth mentioning that previous reports have shown that VP4 associates with microtubules in infected cells (2); however, in our hands, treatment of cells with nocodazole, an agent that disrupts microtubule organization, affected neither the production of viral progeny nor the exit of rotavirus (data not shown).

We found that a fraction of the rotavirus particles in the cell medium were associated with cellular membranes, in agreement with recent reports that have shown that naked viruses can be released from infected cells within vesicles (22, 40–42), although in the case of rotavirus the type of association that the viral particles have with membranous structures remains to be determined. In this regard, Barreto et al. reported that the structural viral protein VP6 is associated with the exosome marker CDC63 *in vitro* and *in vivo* (43), and the same group described that rotavirus infection increases the release of extracellular vesicles (44). While electron microscopy examination of polarized, rotavirus-infected Caco-2 cells did not reveal the presence of extracellular virus within vesicles (15), in this work we found that about 50 to 60% of the extracellular virus was associated with low-density fractions in iodixanol flotation gradients, and the infectivity of the floating virus increased after treatment with Triton X-100, suggesting that a significant fraction of infectious virus is protected by membranous structures.

It has been proposed that in Caco-2 cells there is a post-ER step of rotavirus assembly, since VP4 does not colocalize with ER markers (14). Also, treatment of rotavirus-infected cells with tunicamycin led to an accumulation of enveloped particles within the ER compartment (45), while the expression, trafficking, and localization of VP4 were unaffected by this treatment (14), indicating that VP4 incorporation into mature virions may occur in an extrareticular space. In this regard, it is of great interest that the VP4 present on the surface of MA104 cells can be incorporated into infectious particles. These observations strongly suggest that in addition to the fully documented rotavirus morphogenesis process of viral particles that occurs in the lumen of the ER, an alternative morphogenetic mechanism for the assembly of the spike protein at an extrareticular stage may exist. We envision the following two possibilities for the assembly of the cell surface-located VP4 into infectious particles: (i) the plasma membrane acts as a platform promoting the final step of virus assembly, which is a common feature in enveloped viruses (46); or (ii) the cell surface VP4 is recycled into intracellular vesicles that are transported to sites where triple-layer particles containing VP7, but not VP4, are already assembled, leading to its incorporation into the virion and the formation of mature, infectious viruses. The latter possibility is supported by the observation that VP4 interacts with Rab5 and PRA1, two proteins involved in vesicular trafficking (4). On the other hand, it has been reported that VP4 may associate with different cellular structures in infected cells (28); in this regard, it is noteworthy that not all of the purified TLPs seemed to have incorporated biotinylated VP4 molecules, supporting the hypothesis that the assembly mechanism for VP4 might occur in two different pathways, one in the lumen of the ER and a second involving the cell surface VP4. We suggest that the TLPs in the ER may represent the pool of virus that remains associated with cellular structures at late times postinfection (28 to 48 hpi in MA104 cells), whereas the released virus is primarily composed of virus that uses an alternative pathway for morphogenesis.

Although the fraction of virus found in the extracellular milieu is small compared to that of virus that remains associated with the cell, even after cell lysis, it may have an important role in the dissemination of the virions *in vitro*, and probably *in vivo*. These findings reinforce the relevance of the actin-dependent mechanism for the cell egress of rotavirus that seems to operate in nonpolarized as well as polarized cells.

MATERIALS AND METHODS

Cell lines and viruses. The rhesus monkey kidney epithelial cell line MA104 was grown in Dulbecco's modified Eagle's medium (DMEM)-reduced serum (Thermo Scientific HyClone, Logan, UT) supplemented with 5% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. Rhesus rotavirus strain RRV and the human rotavirus strain Wa were obtained from H. B. Greenberg (Stanford University, USA), and bovine rotavirus UK was obtained from D. R. Snodgrass (Moredun Research Institute, Edinburgh, United Kingdom). All rotavirus strains were grown as described previously (47). Before infection, rotavirus cell lysates were activated with trypsin (10 µg/ml) (Gibco Life Technologies, Carlsbad, CA) for 30 min at 37°C. Purified TLPs of RRV were obtained by use of CsCl isopycnic gradients as reported previously (47).

Antibodies and reagents. Rabbit polyclonal sera to purified rotavirus particles (anti-TLPs) were produced in our laboratory. Monoclonal antibody (MAb) 2G4, specific for VP5, was donated by H. B. Greenberg, and MAb HS2, directed to VP5, was previously reported (27). Goat anti-mouse coupled to Alexa Fluor 568 was obtained from Molecular Probes (Eugene, OR), and a horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody was purchased from PerkinElmer Life Sciences (Boston, MA). Cytochalasin D, latrunculin B, jasplakinolide (JAS), and the cholera toxin B subunit conjugated to horseradish peroxidase were obtained from Sigma (St. Louis, MO).

Treatment of MA104 cells with actin inhibitors. Confluent monolayers of MA104 cells grown in 96-well plates were washed with Eagle's minimum essential medium (MEM) and infected with the RRV, Wa, or UK strain (at an MOI of 3). The virus was adsorbed to cells for 60 min at 37°C. After the adsorption period, the inoculum was removed and MEM was added. At 4 hpi or at different times (as indicated), the cells were treated or not with cytochalasin D (10 mM), latrunculin B (1 µM), or JAS (1 µM). Dimethyl sulfoxide (DMSO) was added to untreated control cells at the concentration used to dissolve the actin inhibitors. The infection was left to proceed for the different indicated periods, with the actin inhibitors maintained in the medium. After the incubation period, the supernatant or cell-associated virus was collected, and the viral titer was quantified by an immunoperoxidase assay of focus-forming units (FFU) as described previously (20).

Cell viability. Cell viability was determined by the following two methods: a lactate dehydrogenase (LDH) release assay, performed by use of a commercial kit (Sigma, St. Louis, MO), and a resazurin reduction assay (Biotium, Inc., CA), performed according to the manufacturer's instructions.

Cell fractionation. MA104 cells grown in 150-cm² flasks were infected with strain RRV (at an MOI of 3), and at 10 hpi, the medium was collected. As a control, CsCl-purified TLPs were mixed with medium from mock-infected cells. Cellular debris was removed by centrifugation at 600 × *g* for 15 min, and the virus present in the supernatant was centrifuged at 150,000 × *g* (Beckman SW55 Ti rotor) for 2 h at 4°C. The pellet was resuspended in TNC (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 10 mM CaCl₂) and mixed with iodixanol (Sigma, St. Louis, MO) to reach a 40% concentration of iodixanol. Discontinuous gradients were prepared by dilution the iodixanol stock solution with TNC. The gradient was set up as follows in 5-ml Beckman SW55 Ti centrifuge tubes, from bottom to top: 1.2 ml of 40% iodixanol (this fraction contained the virus present in the supernatant), 1.5 ml of 35% iodixanol, 0.8 ml of 30% iodixanol, 0.8 ml of 20% iodixanol, and 0.8 ml of 15% iodixanol. The gradient was centrifuged at 150,000 × *g* for 4 h at 4°C, and fractions of 400 µl were collected from the top of the tube. Lipid rafts were characterized by density gradient centrifugation essentially as described previously (48). Eleven fractions were collected from the top of the tube, and the proteins present in each fraction were precipitated with methanol-chloroform-distilled water as reported previously (49).

Cell surface biotinylation. Cells treated or not with JAS were infected with strain RRV (MOI = 3). At 6 hpi, the cells were washed three times with ice-cold phosphate-buffered saline (PBS) and incubated with 0.5 mg of NHS-LC-biotin (Pierce, Rockford, IL) in biotinylation buffer (10 mM triethanolamine, pH 9, 150 mM NaCl, 0.1 mM CaCl₂, 1 mM MgCl₂) for 30 min on ice. Cells were then washed three times with ice-cold PBS, and free biotin was blocked with 100 mM glycine in PBS for 10 min. To analyze the expression of VP4 on the cell surface, cells were lysed with RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and 1% aprotinin) for 10 min, and the lysates were incubated with 20 µl of streptavidin magnetic beads (New England BioLabs, Ipswich, MA) for 1 h at room temperature. The streptavidin beads were washed three times for 5 min each with RIPA buffer, once with a 1:1 mixture of RIPA and TNE (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA), and once with TNE. The biotinylated proteins were resuspended in Laemmli sample buffer, resolved by 10% SDS-PAGE, and subjected to immunoblot analysis. To analyze the incorporation of biotinylated VP4 into TLPs, infected cells were biotinylated as described above, and the cells were then washed three times with 100 mM glycine and three times with MEM and incubated at 37°C for 6 h. After this incubation period, the biotinylated cells were lysed and TLPs were purified twice by use of CsCl gradients as described previously (47).

Immunofluorescence assay. RRV-infected cells grown on coverslips were treated or not with JAS and, at different times postinfection, were fixed with 2% paraformaldehyde (PFA) in PBS for 20 min and permeabilized by incubation with 0.5% Triton X-100 in blocking buffer (1% bovine serum albumin in PBS with 50 mM NH₄Cl) for 15 min. The samples were incubated with primary and secondary antibodies for

1 h each at room temperature in blocking buffer. Nuclei were stained with 30 nM DAPI (4',6-diamidino-2-phenylindole) for 15 min. Coverslips were mounted on glass slides by use of Citifluor AF100 antifade solution (Citifluor Ltd., London, United Kingdom), and the samples were observed under a Zeiss Axioskop 2 fluorescence microscope coupled to a digital camera (Photometrics Cool Snap HQ).

Immunoblot analysis. Cells lysed in Laemmli sample buffer or protein samples precipitated from density gradients were denatured by boiling for 5 min. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat dry milk in PBS and incubated with primary antibodies diluted in PBS containing 5% nonfat dry milk, followed by incubation with species-specific, horseradish peroxidase-conjugated secondary antibodies. The peroxidase activity was detected by using Western Lightning Plus chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instructions.

Transmission electron microscopy. MA104 cells were seeded at 1×10^5 cells in a 2-cm² well onto sapphire discs and infected with RRV at an MOI of 25 viroplasm-forming units (VFU)/ml, as described previously (50, 51). At 6 hpi, MEM was replaced by MEM containing 0.5 μ M JAS, and at 10 hpi, the sapphire discs were collected, fixed with 2.5% glutaraldehyde in 100 mM Na/K-phosphate buffer, pH 7.4, for 1 h at 4°C, and then kept in 100 mM Na/K-phosphate buffer overnight at 4°C. Afterward, samples were postfixed in 1% osmium tetroxide in 100 mM Na/K-phosphate buffer for 1 h at 4°C, dehydrated in a graded ethanol series starting at 70%, followed by two changes in acetone, and embedded in Epon. Ultrathin sections (60 to 80 nm) were cut and stained with uranyl acetate and lead citrate.

For the staining of biotinylated TLPs with streptavidin-gold, freshly purified TLPs from a CsCl gradient were dialyzed overnight at 4°C in TNC buffer. TLPs were adsorbed for 10 min on carbon-coated Parlodion films mounted on 300-mesh/in. copper grids (EMS). Samples were washed once with water, fixed in 2.5% glutaraldehyde in 100 mM Na/K-phosphate buffer, pH 7.0, for 10 min at room temperature, and washed twice with PBS before incubation with 10 μ l streptavidin conjugated to 10-nm colloidal gold (Sigma-Aldrich, Inc.) for 2 h at room temperature. To separate unconjugated streptavidin from streptavidin conjugated to colloidal gold, the streptavidin-gold conjugate was treated as described previously (52) prior to use. The viral particles were further washed three times with water and stained with 2% phosphotungstate, pH 7.0, for 1 min at room temperature. Samples were analyzed in a transmission electron microscope (CM12; Philips, Eindhoven, The Netherlands) equipped with charge-coupled device (CCD) cameras (Ultrascan 1000 and Orius SC1000A; Gatan, Pleasanton, CA, USA) at an acceleration voltage of 100 kV.

Statistical analysis. The statistical significance of differences was determined by one-way analysis of variance (ANOVA) with Tukey's multiple-comparison posttest, using GraphPad Prism 5.0 (GraphPad Software Inc.).

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