

The Spike Protein VP4 Defines the Endocytic Pathway Used by Rotavirus To Enter MA104 Cells

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Rotaviruses are internalized into MA104 cells by endocytosis, with different endocytic pathways used depending on the virus strain. The bovine rotavirus UK strain enters cells through a clathrin-mediated endocytic process, while the simian rhesus rotavirus (RRV) strain uses a poorly defined endocytic pathway that is clathrin and caveolin independent. The viral surface protein VP7 and the spike protein VP4 interact with cellular receptors during cell binding and penetration. To determine the viral protein that defines the mechanism of internalization, we used a panel of UK × RRV reassortant viruses having different combinations of the viral structural proteins. Characterization of the infectivities of these reassortants in MA104 cells either transfected with a small interfering RNA (siRNA) against the heavy chain of clathrin or incubated with hypertonic medium that destabilizes the clathrin coat clearly showed that VP4 determines the pathway of virus entry. Of interest, the characterization of Nar3, a sialic acid-independent variant of RRV, showed that a single amino acid change in VP4 shifts the route of entry from being clathrin dependent to clathrin independent. Furthermore, characterizations of several additional rotavirus strains that differ in their use of cellular receptors showed that all entered cells by clathrin-mediated endocytosis, suggesting that diverse VP4-cell surface interactions can lead to rotavirus cell entry through this endocytic pathway.

Rotaviruses are the principal worldwide etiologic agents of severe viral gastroenteritis in infants and children, causing more than half a million deaths every year (1). These viruses belong to the family *Reoviridae* and, as such, are nonenveloped and have a genome composed of 11 segments of double-stranded RNA that encodes six structural proteins (VP1 to VP4, VP6, and VP7) and six nonstructural proteins (NSP1 to NSP6). The virus particles are formed by three concentric layers of proteins. The innermost layer is made up of VP2, which together with the viral genome and small amounts of VP1 and VP3 forms the core of the particle. The intermediate layer is formed by VP6, and the outermost layer is composed of trimers of the VP7 glycoprotein that form the smooth surface of the virus, from which the VP4 trimers that form the viral spikes project, thus forming the mature infectious triple-layered particles (TLPs) (2). Trypsin treatment of rotavirus is essential for virus cell entry; what results is the specific cleavage of VP4 to yield the cleavage products VP8 and VP5 (3–7). Analysis of the crystal structure of these domains has shown that VP8 forms the head, while VP5 forms the body and foot of the VP4 spike (8, 9).

Several cell molecules thought to function as virus receptors have been shown to interact with the virus surface proteins during the early steps of virus infection (10). VP7 binds integrins $\alpha v\beta 3$ and $\alpha x\beta 2$ (11, 12), while VP5 has a conserved Asp-Gly-Glu (DGE) binding motif for integrin $\alpha 2\beta 1$ (11, 13–16) and also interacts with heat shock cognate protein 70 (hsc70) (17, 18). While the interaction with integrins has been reported to be strain dependent, all viruses tested have been shown to require hsc70 for cell infection (19). The VP8 domain of some virus strains contains a binding domain for terminal sialic acid (SA) (20) that is used for cell attachment of the virus. The infectivities of these viruses are thus inhibited by treatment of the cell with neuraminidase (NA); these are referred to as NA-sensitive strains (21). In contrast, most virus strains are NA resistant (22, 23), and it was recently shown that the VP8 domain of NA-resistant rotaviruses can interact with subterminal SA, which is not susceptible to cleavage by NA, or

with carbohydrate moieties related to human blood antigens (24–28). The initial interaction of NA-sensitive viruses with SA has been shown to be nonessential, since variants with a single amino acid change in the SA-binding domain of VP8, which bypass the VP8-SA interaction step, can be isolated (29–31). In the case of the NA-sensitive rhesus rotavirus (RRV), its NA-resistant variant Nar3 has been shown to attach to cells directly by interacting with integrin $\alpha 2\beta 1$ (15, 16).

Ultimately, the interactions described are believed to lead to rotavirus internalization by endocytosis (19, 32–34). Rotaviruses can be internalized into MA104 cells using different endocytic pathways depending on the virus strain. Bovine rotavirus strain UK, an integrin-independent NA-resistant strain that seems to attach to subterminal SA, enters cells by clathrin-mediated endocytosis, while the integrin-dependent NA-sensitive simian rotavirus strain RRV uses a poorly defined endocytic pathway that is different from macropinocytosis and is independent of clathrin and caveolin but depends on dynamin II, the small GTPases RhoA and Cdc42, actinin-4, and the presence of cholesterol on the cell surface (19, 32) (D. Silva-Ayala, M. Gutiérrez, T. López, N. Perri-mon, S. López, and C. F. Arias, submitted for publication).

Given the ample differences observed among rotavirus strains in regard to receptor usage and the two cell entry pathways described above, in this study we sought a better understanding of the strain-dependent variations in the rotavirus entry process by determining whether there are strain-dependent differences in clathrin dependence during entry, which of the two outer-layer proteins determine

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this choice, and whether the choice is dictated by the type of glycans used during initial attachment. Using a collection of UK × RRV reassortant viruses, we found that the spike protein VP4 is sufficient to define the endocytic pathway used. In addition, characterization of the RRV Nar3 mutant showed that a single amino acid change in VP4 can shift clathrin-dependent endocytosis to a different endocytic route. By testing different rotavirus strains, we also showed that diverse virus-cell surface interactions can all lead to rotavirus cell entry through clathrin-mediated endocytosis.

MATERIALS AND METHODS

Cell culture and viruses. The rhesus monkey epithelial cell line MA104 (ATCC) was grown in advanced Eagle's minimal essential medium (MEM) (Invitrogen) supplemented with 4% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. The simian rotavirus strain RRV (G3P[3]), bovine rotavirus strain UK (G6P[5]), and rotavirus reassortants UK × RRV used in this work, with the exception of reassortant 85-2, have been described previously (35). Reassortant 85-2 was generated using the same procedure used for the other UK × RRV reassortants (35). RRV variant Nar3 and its revertant clones 14 and 18 (rNar3-14 and rNar3-18) have been described previously (31, 36). Simian rotavirus strain SA11-4S (clone 3) (G3P[2]) was obtained from M. K. Estes (Baylor College of Medicine, Houston, TX). Human rotavirus strains Wa (G1P[8]) and DS-1 (G2P[4]) were obtained from H. B. Greenberg (Stanford University, Stanford, CA), and rotavirus WI-61 (G9P[8]) was obtained from F. H. Clark (Wistar Institute, Philadelphia, PA; through M. K. Estes). The porcine strain YM (G11P[7]) was isolated in our laboratory (37). All rotavirus strains were propagated in MA104 cells as described previously (38). Rotavirus cell lysates were activated with trypsin (10 µg/ml) for 30 min at 37°C.

Reagents and antibodies. Sucrose, EGTA, neuraminidase (NA) from *Arthrobacter ureafaciens*, and glycoprotein A (GpA) were purchased from Sigma-Aldrich (Saint Louis, MO). Trypsin was purchased from Gibco-BRL. The small interfering RNA (siRNA) against the luciferase gene and the SMARTpool siRNA against the clathrin heavy chain (CHC) were purchased from Dharmacon Research (Lafayette, CO). A monoclonal antibody (MAb) to CHC was purchased from Thermo Fisher Scientific (USA). Horseradish peroxidase-conjugated goat anti-rabbit polyclonal and anti-mouse antibodies were purchased from PerkinElmer Life Sciences (Boston, MA). The goat and rabbit polyclonal sera, raised against purified rotavirus TLPs, and the rabbit anti-vimentin serum against recombinant vimentin (α-Vim) were produced in our laboratory.

Cell treatments. Transfection of siRNAs into MA104 cells was performed in 96-well plates using a reverse transfection method as described previously (19). For the sucrose cell treatment, confluent monolayers of MA104 cells grown in 96-well plates were washed twice with MEM and pretreated with 250 mM sucrose as described previously (19). For NA cell treatment, confluent monolayers of MA104 cells grown in 96-well plates were washed twice with MEM and pretreated with 80 mU/ml of *A. ureafaciens* NA as described previously (36). After activation with trypsin, the viral cell lysates were incubated with GpA for 30 min at a final concentration of 10 µg/ml as described previously (36), and this mixture was used to infect cells.

Infectivity assays. MA104 cells were washed twice with MEM and infected at a multiplicity of infection (MOI) of 0.02 using cell lysates of the indicated rotavirus strain. After 1 h of adsorption, the virus was removed, and the cells were washed twice and incubated with MEM for 14 additional hours. The cell monolayers were fixed, and the infected cells were revealed by an immunoperoxidase focus detection assay as described previously (38). In the sucrose infectivity assays, after the adsorption period and before washing the cells with MEM, the cells were washed twice with EGTA-phosphate-buffered saline (PBS) as described previously (19). When a combined approach was used, cells were transfected with the indicated siRNA and subsequently treated with NA.

TABLE 1 Capsid proteins of UK × RRV reassortants employed in this study

Virus	Origin of ^a :					
	VP1	VP3	VP2	VP6	VP7	VP4
UK	U	U	U	U	U	U
RRV	R	R	R	R	R	R
UK × RRV rotavirus reassortants						
9 (9-8-1) ^b	U	U	U	U	R	U
13 (13-1-1)	R	R	R	U	R	R
19 (19-1-1)	R	R	R	R	R	U
20 (20-1-1)	U	U	R	R	U	U
21 (21-1-1)	U	U	U	R	U	U
25 (25-2-1)	R	R	R	U	R	R
27 (27-2-1)	R	R	R	U	R	R
85 (85-2)	U	R	R	R	U	R

^a U indicates that the protein is of UK origin, and R indicates that the protein is of RRV origin.

^b Numbers in parentheses are the original reported names of the reassortant viruses 35.

Binding assays. MA104 cells grown in 96-well plates and transfected with siRNAs were treated with NA as described above; the cells were then washed twice and incubated with MEM for 30 min at 37°C, and virus binding was assayed as described previously (39).

Immunoblots. Cells were lysed in Laemmli sample buffer and denatured by boiling for 5 min. The cell lysates were then subjected to SDS-10% PAGE and transferred to Immobilon NC (Millipore) membranes. The membranes were processed for immunoblotting as described previously (19).

Statistical analysis. Statistical significance was evaluated by using a one-way analysis of variance (ANOVA) test followed by Tukey's multiple-comparison posttest using GraphPad Prism 5.0 (GraphPad Software, Inc.). *P* values of less than 0.05 were considered significant.

RESULTS

VP4 defines the clathrin-dependent internalization of bovine rotavirus strain UK. Rotavirus strain UK enters cells by clathrin-mediated endocytosis, while the simian rotavirus strain RRV uses an as-yet-undefined endocytic pathway. As an initial approach to identifying which viral protein is associated with the endocytic pathway used by these viruses, we evaluated the sensitivities of a collection of UK × RRV rotavirus reassortants having different combinations of structural proteins (Table 1) to cell treatments known to impair clathrin-mediated endocytosis. Treatment of cells with a hypertonic medium (250 mM sucrose) results in the dissociation of clathrin vesicles from the plasma membrane (40, 41). As reported previously (19), the infectivity of RRV was not affected under these conditions, while the infectivity of UK decreased by about 40%. Of interest, the infectivities of reassortants that have the VP4 protein from the bovine rotavirus (reassortants 9, 19, 20, and 21) also decreased to about the same level, while the infectivities of reassortants that contain the VP4 protein of RRV (viruses 13, 25, 27, and 85) were not affected (Fig. 1). These results strongly suggest that VP4 is the protein that confers sensitivity to hypertonic medium.

To confirm the previous results, we evaluated the infectivity of the panel of UK × RRV reassortants in MA104 cells in which the expression of the clathrin heavy chain (CHC) was knocked down by RNA interference (RNAi). In these assays, the infectivity of rotavirus strain UK was inhibited by about 75% in cells transfected with the siRNA against CHC compared to cells transfected with an irrelevant siRNA, while the infectivity of RRV remained

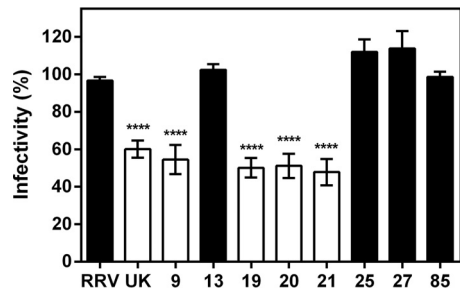


FIG 1 Effect of hypertonic medium on the infectivities of the UK × RRV reassortants. MA104 cells were incubated with sucrose at 250 mM in MEM for 10 min at 37°C before they were infected with the indicated viruses at an MOI of 0.02 for 1 h at 37°C in the presence of the same hypertonic medium. After the adsorption period, the virus inoculum was removed, the cells were washed twice with 5 mM EGTA, and the infection was left to proceed for 14 h at 37°C. The infected cells were fixed and immunostained as indicated in Materials and Methods. Data are expressed as the percent infectivity of each virus observed in nontreated cells, which represent 100% infectivity. Black bars, RRV and reassortants bearing RRV VP4; white bars, UK and reassortants bearing UK VP4. The arithmetic means ± standard deviation of three independent experiments performed in duplicate are shown. The asterisks indicate significant differences between the infectivity of each virus in mock- and sucrose-treated cells ($P \leq 0.001$).

unaffected (Fig. 2A), as described previously (19). The infectivities of the reassortant viruses with the VP4 protein of UK origin (reassortants 9, 19, 20, and 21) were significantly decreased by this treatment, while reassortants 13, 25, 27, and 85, which have the VP4 of RRV, behaved like the RRV parental virus. Of particular interest, the infectivity of reassortant 19 that has a UK VP4 in an RRV capsid protein background was sensitive to CHC silencing. It

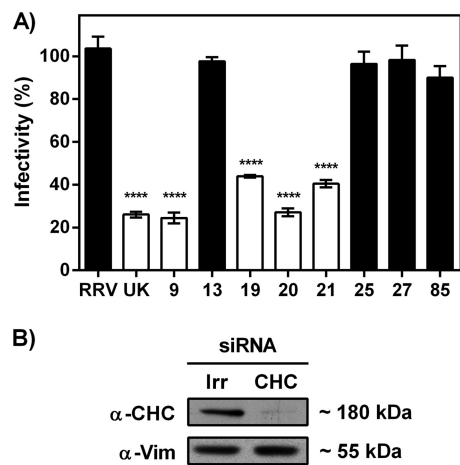


FIG 2 Infectivities of UK × RRV reassortants in CHC knocked-down cells. (A) MA104 cells transfected with a SMARTpool siRNA against the clathrin heavy chain (CHC) were infected with the indicated viruses at an MOI of 0.02. At 14 hours postinfection (hpi), the cells were fixed and immunostained as described in Materials and Methods. Data are expressed as the percent infectivity of each virus compared to infection of cells transfected with an irrelevant siRNA (luciferase siRNA), which represents 100% infectivity. Black bars, RRV and reassortants bearing RRV VP4; white bars, UK and reassortants bearing UK VP4. The arithmetic means ± standard deviation of three independent experiments performed in duplicate are shown. The asterisks indicate significant differences between the infectivities of each virus in irrelevant and CHC siRNA-transfected cells ($P \leq 0.001$). (B) Representative immunoblot for determination of the abundance of CHC in MA104 cells transfected with either irrelevant or CHC siRNAs (Irr and CHC, respectively). Vimentin (Vim) was used as a loading control. The antibodies used are indicated.

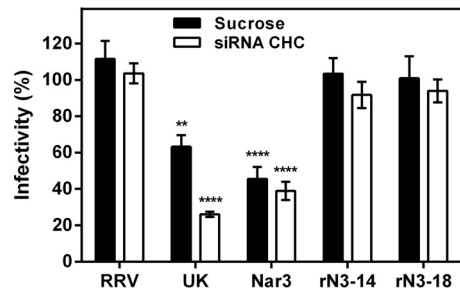


FIG 3 Effects of cell treatments that affect clathrin-dependent endocytosis on the infectivities of RRV variants. MA104 cells treated with sucrose or transfected with the siRNA against CHC were infected with the indicated viruses at an MOI of 0.02. Cells were fixed and immunostained at 14 hpi as described in Materials and Methods. Data are expressed as the percent infectivity of each virus compared to their infectivity under mock sucrose conditions or in cells transfected with an irrelevant siRNA. The arithmetic means ± standard deviation of three independent experiments performed in duplicate are shown. **, $P < 0.01$, and ****, $P \leq 0.0001$, for statistically significant differences between the infectivities of each virus under experimental and control conditions. Revertant viruses rNar3-14 and rNar3-18 are denoted as rN3-14 and rN3-18.

is also of note that even though there was not a single-gene reassortant virus with an RRV VP4 protein in a UK background, all reassortants with an RRV VP4 were resistant to CHC knockdown, suggesting that these viruses enter the cells through the endocytic pathway used by the parental RRV virus, guided by the spike protein VP4. To discard the possibility that silencing of CHC had affected the membrane surface availability of an unknown UK attachment receptor, we tested the binding of UK and RRV strains to MA104 cells transfected with the siRNA against CHC. The attachment of both rotavirus strains was not altered in these cells compared to control cells transfected with an irrelevant siRNA (data not shown). Altogether, these results indicate that UK VP4 alone is sufficient to direct the entry of rotavirus into MA104 cells through clathrin-mediated endocytosis.

A single amino acid change in VP4 defines the pathway of rotavirus cell entry. RRV binds to MA104 cells through the interaction of an SA-binding domain in VP8, located at the tip of the spike (9). This binding, however, is not essential for infectivity of the virus, since RRV variants that bypass this interaction and bind instead to cells by interacting with integrin $\alpha 2 \beta 1$ through the VP5 domain of VP4 have been isolated (36). Sequence characterizations of these NA-resistant (Nar) variants showed the presence of three amino acid changes in VP4 with respect to the wild-type virus (L37P, K187R, and Y267C), while the RRV phenotypic reversion of the Nar mutants occurred through a single amino acid change (the genetic reversion of R187 back to K in the VP8 subunit of VP4) (31). To assess if a change in the cell surface molecule used by the virus to anchor to the cell surface might influence the cell entry pathway, we characterized the effect of hypertonic medium and CHC dependence on the infectivity of the RRV NA-resistant variant Nar3 and its NA-sensitive revertants. As shown in Fig. 3, the infectivity of Nar3 was inhibited to a level similar to that of rotavirus strain UK by both sucrose treatment and the siRNA against CHC, while the infectivities of the two revertant viruses, rNar3-14 and rNar3-18, remained unaffected. These results suggest that in contrast to its parental RRV strain, variant Nar3 enters MA104 cells through a clathrin-mediated pathway as a result of a single amino acid change in the VP8 domain of VP4.

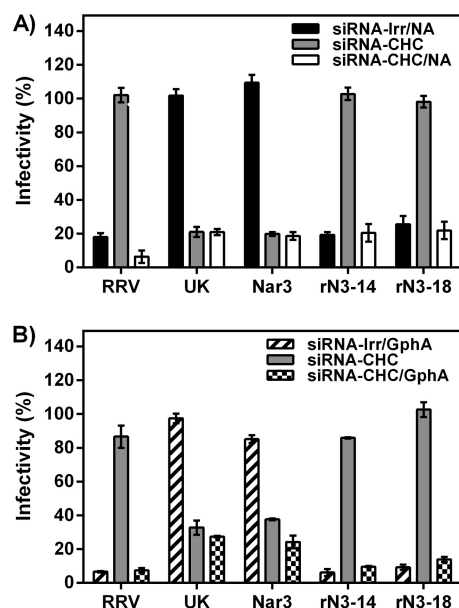


FIG 4 Effects of NA treatment and GphA on the infectivities of RRV variants in CHC-silenced cells. (A) MA104 cells transfected with an irrelevant siRNA or an siRNA against CHC were treated or not with NA (80 μ M/ml for 1 h) and infected with the indicated RRV variants. Cells were fixed and immunostained at 14 hpi as described in Materials and Methods. Data are expressed as the percent infectivity of each virus compared to its infectivity in cells transfected with the irrelevant siRNA but not treated with NA. (B) MA104 cells transfected with an irrelevant siRNA or an siRNA against CHC were infected with the indicated viruses that had been incubated with GphA. Cells were fixed and immunostained at 14 hpi as described in Materials and Methods. Data are expressed as the percent infectivity of each virus compared to its infectivity in the absence of GphA in cells transfected with an irrelevant siRNA. The arithmetic means \pm standard deviation of three independent experiments performed in duplicate are shown. Revertant viruses rNar3-14 and rNar3-18 are denoted as rN3-14 and rN3-18.

The interaction of VP8 with sialic acid is not necessary for clathrin-dependent cell entry of Nar3. The cell attachment of Nar3 is mediated by the $\alpha 2\beta 1$ integrin-binding motif DGE, present in the VP5 domain of VP4 (15, 16). However, although Nar3 uses $\alpha 2\beta 1$ integrin instead of terminal SA to attach to MA104 cells, the VP8 domain of this RRV variant still maintains the ability to interact with SA residues (36). To evaluate the potential role of the VP8-SA interaction in the entry of rotavirus through clathrin-dependent endocytosis, we assayed the infectivities of RRV and Nar3 in cells treated with NA that had been previously transfected with the siRNA against CHC. As expected, the infectivity of RRV decreased in NA-treated cells transfected with an irrelevant siRNA or with the siRNA against CHC (Fig. 4A). The infectivity of Nar3, like that of UK, was inhibited only in MA104 cells in which the expression of CHC was knocked down, regardless of whether the cells had been treated with NA (Fig. 4A); the infectivities of the revertant viruses, rNar3-14 and rNar3-18, were similar to that of RRV under these conditions.

These results were confirmed by evaluating the infectivities of the viruses in the presence of GphA. This highly sialylated protein binds to the SA-binding domain of RRV and Nar3 but only inhibits the infectivity of the parental virus (36). As reported, in cells transfected with an irrelevant siRNA, preincubation with GphA decreased the infectivities of RRV and the Nar3 revertant viruses by more than 90%, while the infectivities of Nar3 and UK were not

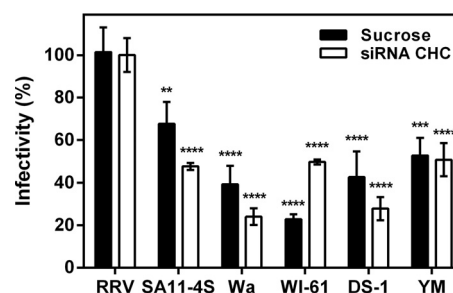


FIG 5 Infectivities of different rotavirus strains in cells with treatments that inhibited clathrin-mediated endocytosis. MA104 cells treated with sucrose or transfected with an siRNA against CHC were infected with the indicated rotavirus strains at an MOI of 0.02. At 14 hpi the cells were fixed and immunostained as described in Materials and Methods. Data are expressed as the percent infectivity of each virus compared to their infectivities under mock sucrose conditions or in cells transfected with an irrelevant siRNA. The arithmetic means \pm standard deviation of three independent experiments performed in duplicate are shown. **, $P < 0.01$, ***, $P \leq 0.001$, and ****, $P \leq 0.0001$, for statistically significant differences between the infectivities of each virus under experimental and control conditions.

significantly affected (Fig. 4B). Of interest, the infectivity of Nar3 was inhibited by about 80% when the virus was preincubated with GphA and used to infect MA104 cells in which the expression of CHC had been silenced. These results indicate that despite having its VP8 domain blocked by GphA, the virus still enters the cell through a clathrin-dependent endocytosis route. The results also confirm that the entry through clathrin-mediated endocytosis is independent of the VP8-SA interaction and seems to be guided instead by the interaction of VP5 with integrin $\alpha 2\beta 1$ or by a subsequent virus-receptor interaction.

Rotavirus strains with different cell receptor requirements enter MA104 cells through a clathrin-mediated pathway. Since VP4 seems to be the protein responsible for the type of endocytosis that rotaviruses use to infect cells, it was of interest to investigate the mode of entry of rotavirus strains that bind to different cell surface molecules and belong to different P genotypes. We evaluated the entry pathway of simian rotavirus strain SA11-4S (P[2]), which binds to cells through SA. We also evaluated the cell entry of the NA-resistant human rotavirus strains Wa (P[8]), WI-61 (P[8]), and DS-1 (P[4]). These strains were recently reported to bind Lewis b and H type 1 histo-blood group antigens, suggesting that they may use these molecules as receptors for cell attachment (27). Additionally, we evaluated the infectivity of the NA-sensitive porcine rotavirus strain YM (P[7]). The infectivities of all these strains were determined in MA104 cells treated with a hypertonic medium or with the siRNA against CHC. Of note, under both conditions and despite the occurrence of binding to the terminal SA on the cell surface, the infectivities of the SA11-4S and YM virus strains were decreased (Fig. 5). Similarly, the infectivities of the human rotavirus strains Wa, WI-61, and DS-1 decreased significantly in cells treated with sucrose and were inhibited between 50% and 75% in cells transfected with the siRNA against CHC compared to cells transfected with an irrelevant siRNA, which represents the 100% infectivity in each case. These findings suggest that different VP4-cell surface interactions can lead to rotavirus cell entry by a clathrin-dependent endocytic process.

DISCUSSION

Rotavirus entry into MA104 cells occurs through endocytosis with the use of different mechanisms depending on the virus strain (19). Taking advantage of the differential endocytic pathways used by the rotavirus UK and RRV strains to enter MA104 cells, we used a panel of UK \times RRV reassortants to determine the viral protein that segregates with the type of endocytosis employed. Analysis of these reassortants showed unambiguously that the spike protein VP4 defines the clathrin-mediated endocytosis entry of UK since the reassortant virus 19, which has a UK VP4 protein in a capsid otherwise formed by RRV proteins, is also clathrin dependent. Even though our collection did not have a single reassortant virus with an RRV VP4 on a UK capsid protein background, the presence of UK VP7 (reassortant 85) and VP6 (reassortants 13, 25, and 27) in viruses that enter cells by a clathrin-independent process, as RRV does, strongly suggests that RRV VP4 also determines the endocytic pathway used by RRV. Although unlikely, the possibility that RRV VP2, present in all viruses with an RRV-like mechanism of entry, might influence the conformation of VP4 and help define the entry route of these reassortant viruses cannot be discounted. Also, the lack of correlation between the G genotype and the mode of entry of the various rotavirus strains characterized in this work suggests that VP7 is not involved in determining the endocytic pathway of rotaviruses other than RRV and UK and that VP4 is most probably the key protein in this process. However, this supposition remains to be directly tested. RRV was the only strain evaluated with a P[3] genotype; thus, it is not possible to determine if entry into the virus by a clathrin-independent route is a particular property of RRV or if it is associated with the P genotype of the virus.

It has been proposed that rotaviruses infect cells by a complex multistep process (10). Several lines of evidence suggest that rotavirus RRV interacts sequentially with several cell surface molecules during its entry into MA104 cells and uses different domains on the virus surface proteins VP4 and VP7 during this process (42). It is thought that the initial contact of RRV with the cell surface is through an SA-containing cell receptor using the VP8 domain of VP4. Gangliosides have been suggested to play this role (43, 44), although recent data suggest that gangliosides could function during rotavirus entry at a postattachment step (45). The initial interaction of VP8 with SA is proposed to induce a subtle conformational change on VP4 that allows the virus to interact subsequently with integrin $\alpha 2\beta 1$ through the DGE motif on VP5 (42). It has been proposed that after this second interaction, VP5 interacts with hsc70 and VP7 interacts with integrins $\alpha \nu \beta 3$ and $\alpha \nu \beta 2$ (11, 12, 15–18, 39). Whether these three latter interactions occur sequentially or alternatively has not been established. In this context, the single amino acid change in the RRV variant Nar3 that confers its NA resistance to infect cells was shown to allow the virus to take a shortcut, bypassing the initial interaction with SA and attaching to the cell surface directly by interacting with integrin $\alpha 2\beta 1$ through VP5 (15, 16). After their interaction with integrin $\alpha 2\beta 1$, both RRV and Nar3 viruses were thought to follow the same entry pathway. However, although subsequent to their interaction with $\alpha 2\beta 1$ both viruses interact with integrins $\alpha \nu \beta 3$ and $\alpha \nu \beta 2$ and with hsc70 (12, 39), the fact that these viruses enter cells by different endocytic routes indicates that the entry processes of rotaviruses might be more complex than thought. Further investigation is required to determine whether subtle differences in the interactions of RRV and Nar3 with one or more of the aforementioned receptors are

responsible for the different endocytic pathways taken by these viruses or whether an additional unidentified cell surface molecule is responsible for their differential cell entry phenotype.

The example of variant Nar3 is not unprecedented, since single or a few amino acid changes in echovirus (46), enterovirus (47–49), coxsackievirus (50–52), and foot-and-mouth disease virus (53–55) have been shown to be sufficient to alter the receptor specificity and the endocytic mode of entry of these viruses. In the case of rotaviruses, however, it seems that the cell surface molecule used to attach to cells does not by itself determine the type of endocytosis pathway employed for virus cell entry. As mentioned before, rotavirus strains RRV, SA11-4S, and YM are all NA sensitive, and yet they enter cells by different endocytic mechanisms. In this regard, it is of interest that the entire $\alpha 3$ - $\alpha 4$ region of integrin $\alpha 2\beta 1$ has been shown to be important for the interaction with human and RRV rotavirus strains, while this region of $\alpha 2\beta 1$ is less important for the interaction with SA11 and Nebraska calf diarrhea virus (NCDV) rotaviruses (13). These data suggest that one possible explanation for the difference in the endocytic pathway used by rotaviruses SA11 and YM, as well as by TFR-41 and that used by RRV, regardless of their sialic acid usage, could be a subtle differential interaction with one of the described receptors or a differential interaction with one additional so-far-unidentified cell surface molecule. In addition, with the exception of RRV, all other rotavirus strains tested (see also reference 19) use clathrin-mediated endocytosis to enter cells, regardless of the P genotype of the virus, the cell attachment receptor used, and whether virus infection is dependent or independent of integrins. These findings suggest that diverse VP4-cell surface interactions can lead to rotavirus cell entry via clathrin-dependent endocytosis.

In summary, in this work we have shown that the spike protein VP4 determines the pathway of cell internalization of the virus. It remains to be determined which one of the several interactions described for this protein, or whether a new VP4-cell interaction not described yet, triggers the specific endocytic process.

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