Different Rotavirus Strains Enter MA104 Cells through Different Endocytic Pathways: the Role of Clathrin-Mediated Endocytosis[▽]

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Rotaviruses, the single most important agents of acute severe gastroenteritis in children, are nonenveloped viruses formed by a three-layered capsid that encloses a genome formed by 11 segments of double-stranded RNA. The mechanism of entry of these viruses into the host cell is not well understood. The best-studied strain, RRV, which is sensitive to neuraminidase (NA) treatment of the cells, uses integrins $\alpha 2\beta 1$ and $\alpha \nu \beta 3$ and the heat shock protein hsc70 as receptors and enters MA104 cells through a non-clathrin-, non-caveolin-mediated pathway that depends on a functional dynamin and on the presence of cholesterol on the cell surface. In this work, using a combination of pharmacological, biochemical, and genetic approaches, we compared the entry characteristics of four rotavirus strains known to have different receptor requirements. We chose four rotavirus strains that represent all phenotypic combinations of NA resistance or sensitivity and integrin dependence or independence. We found that even though all the strains share their requirements for hsc70, dynamin, and cholesterol, three of them differ from the simian strain RRV in the endocytic pathway used. The human strain Wa, porcine strain TFR-41, and bovine strain UK seem to enter the cell through clathrin-mediated endocytosis, since treatments that inhibit this pathway block their infectivity; consistent with this entry route, these strains were sensitive to changes in the endosomal pH. The inhibition of other endocytic mechanisms, such as macropinocytosis or caveola-mediated uptake, had no effect on the internalization of the rotavirus strains tested here.

Endocytosis is a cellular process that involves the formation of a vesicle whose cargo is transported from the extracellular milieu to the interior of the cell. Several endocytic pathways have been described, and all of them have been shown to be used by viruses during cell entry. These pathways include clathrin-mediated endocytosis, uptake via caveolae, macropinocytosis, phagocytosis, and a novel non-clathrin-, non-caveola-mediated pathway that is currently not well characterized (32). While detailed information about the entry of several enveloped viruses is now available (4, 35, 49, 53, 56), the mechanism by which nonenveloped viruses enter cells is not well understood. Two general mechanisms have been proposed to be used by these viruses to reach the cell's cytoplasm: direct penetration at the cell surface, during which the viral particles are directly translocated from the external milieu into the cytoplasm, or internalization through endocytic processes (55).

Rotaviruses, members of the family *Reoviridae*, are the leading etiologic agent of viral gastroenteritis in infants and young children worldwide, being responsible for an estimated 500,000 deaths each year (41). These nonenveloped viruses are formed by three concentric layers of protein that surround the viral genome, formed by 11 segments of double-stranded RNA. The outermost layer of the virion is formed by two proteins, VP4 and VP7, which are involved in the early interactions of the

virus with its host cell (7, 27). VP4 is involved in receptor binding and cell penetration. The role of VP7 is less clear, although it has been shown that it interacts with the cell surface molecules at a postattachment step (17). After binding to the cell surface, the virus penetrates the plasma membrane to productively infect the cell. This penetration depends on the trypsin treatment of the virus, which results in the specific cleavage of VP4 to polypeptides VP8 and VP5. This cleavage promotes VP4 rearrangements in the viral particles that rigidify the spikes (7, 11).

Despite the fact that, *in vivo*, rotaviruses primarily infect the mature enterocytes of the small intestine, studies of the infection of this type of cells have been limited due to the lack of established intestinal cell lines of small intestine origin. Given the absence of a better model, most of the studies on the entry and replication cycle of rotavirus have been conducted either in the epithelial monkey kidney cell line MA104 or in the human colon carcinoma cell line Caco-2, which are highly permissible to these viruses.

Using as a model MA104 cells and the simian rotavirus RRV, we have proposed that rotavirus cell entry is a complex multistep process that involves the two virus surface proteins and several cell receptors, including sialic acids, gangliosides, integrins $\alpha 2\beta 1$, $\alpha 4\beta 1$, $\alpha \nu \beta 3$, and $\alpha \nu \beta 2$, and the heat shock cognate protein hsc70 (22). We have also shown that depletion of cholesterol from the cellular membrane severely impairs the infectivity of rotavirus (19, 50) and have suggested that sphingolipid- and cholesterol-enriched membrane lipid microdomains might be involved in rotavirus cell entry, since the virus and its receptors associate with these domains at early times

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TABLE 1. Cellular receptor requirements of different rotavirus strains

Strain	Origin	Neuraminidase sensitivity	Integrin dependent	References
RRV	Simian	Yes	Yes	5, 6, 17
TFR-41	Porcine	Yes	No	6, 17
UK	Bovine	No	No	6, 17
Wa	Human	No	Yes	5, 17, 34

during infection (23). However, there are some rotavirus strains that may not use all these molecules; some rotavirus strains are resistant to the neuraminidase (NA) treatment of the cell, and thus, they have been classified as NA resistant (6, 34). Additionally, the infectivity of some viral strains is not blocked by anti-integrin antibodies, suggesting the existence of rotavirus strains that are integrin independent (Table 1) (17).

The precise mechanism utilized by rotavirus to enter the cell is, however, not yet defined. Recently, we reported that the entry of the simian rotavirus strain RRV is independent of clathrin- and caveola-mediated endocytosis; however, it is dependent on dynamin (a protein involved in the scission of the endocytic vesicles from the cellular membrane) and requires the presence of cholesterol in the cell membrane (50). In this work, using a combination of pharmacological, biochemical, and genetic approaches, we compared the entry characteristics of four rotavirus strains known to have different receptor requirements. We found that all the strains tested share the requirement for hsc70, cholesterol, and dynamin. Unexpectedly, we found that there were differences in the type of endocytic route utilized by three of the strains compared to that of simian strain RRV. Bovine strain UK, porcine strain TFR-41, and human strain Wa more likely enter the cell through a clathrin-dependent mechanism, since treatments that inhibit this process block the infectivity of these rotavirus strains; in contrast, the entry of RRV, as previously shown (50), is independent of this pathway. The inhibition of other endocytic mechanisms, such as macropinocytosis or caveola-mediated uptake, had no effect on the entry of the rotavirus strains tested here.

MATERIALS AND METHODS

Cells and viruses. The rhesus monkey epithelial cell line MA104 was grown in Advanced DMEM (Dulbecco's modified Eagle's medium) (Invitrogen, Carlsbad, CA) supplemented with 3% fetal bovine serum (FBS) and was used for all experiments carried out in this work. Rhesus rotavirus strain RRV and the human strain Wa were obtained from H. B. Greenberg (Stanford University, Stanford, CA), bovine rotavirus UK was donated by D. R. Snodgrass (Moredun Research Institute, Edinburgh, United Kingdom), and porcine strain TFR-41 was obtained from I. Holmes (University of Melbourne, Victoria, Australia). All rotavirus strains were grown in MA104 cells as described previously (40). Double-layered rotavirus particles (DLPs) from different strains were purified by CsCl density gradient as reported previously (14). Reovirus type 1 was kindly provided by C. Ramos (CISEI, National Institute of Public Health, Cuernavaca, Mexico). Simian virus 40 (SV40) was obtained from L. Gutiérrez (National Institute of Public Health, Cuernavaca, Mexico).

Antibodies and reagents. Monoclonal antibodies (MAbs) to clathrin heavy chain, hsc70, and hsp70 were purchased from ABR Antibodies (Golden, CO). MAbs against the large antigen of SV40 (anti-SV40 TAG) and antihemagglutinin (anti-HA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Tetramethylrhodamine-conjugated dextran (TMR-dextran) and Alexa 488- and 568-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated goat anti-rabbit polyclonal

antibody was from Perkin Elmer Life Sciences (Boston, MA). Rabbit polyclonal antibodies to purified rotavirus particles (anti-TLPs), to rotavirus nonstructural protein (anti-NSP2), to reovirus, and to vimentin were produced in our laboratory. Methyl- β -cyclodextrin (M β CD), sucrose, bafilomycin A1, DMA [5-(*N,N*-dimethyl)amlioride hydrochloride], and water-soluble cholesterol (BioReagent product no. C4951) were purchased from Sigma (St. Louis, MO); ammonium chloride was purchased from J.T. Baker (Phillipsburg, NJ).

Infectivity assay. Confluent MA104 cells in 96-well plates (for rotavirus infection) or 24-well plates (for SV40 infection) were washed twice with Eagle's minimum essential medium (MEM), and then approximately 2,000 focus-forming units (FFU) of rotavirus or reovirus or 6,000 FFU of SV40 were adsorbed to cells for 60 min at 37°C. After the adsorption period, the virus inoculum was removed, MEM was added, and the infection was left to proceed for 15 h for reovirus and rotavirus strains or 18 h for SV40 at 37°C. Infected cells were detected by an immunoperoxidase FFU assay using a rabbit hyperimmune serum directed to triple-layered particles (TLPs) (for RRV, UK, and TFR-41), a hyperimmune serum to rotavirus Wa, or a rabbit antireovirus antibody, as described previously (8). Briefly, cell monolayers were fixed with 80% acetone in phosphate-buffered saline (PBS), and intracellular viral antigen was detected by antirotavirus or antireovirus polyclonal antibodies followed by a peroxidase-conjugated goat anti-rabbit polyclonal antibody as described earlier (8). The FFUs were counted in a VisiLab 1000 station (Biocom, Les Ulises, France) as reported previously (19). SV40-infected cells were detected by a fluorescence focus assay using an anti-SV40 TAG MAb as described previously (52).

Kinetics of rotavirus cell entry. To measure the rate of rotavirus cell entry, confluent monolayers of MA104 cells grown in 96-well plates were washed twice with cold MEM and left to chill on ice. Then, approximately 2,000 FFUs of each rotavirus strain were added per well, and the viruses were allowed to bind on ice for 1 h. After this time, unbound viruses were washed, warmed MEM was added, and the plates were placed over a 37°C water bath. At the time points indicated (see Fig. 1), the medium in the wells was aspirated, and viral particles still present on the cell surface were detached by two quick washes with 3 mM EGTA in PBS, followed by incubation in warm MEM. After reaching the last time point of the entry measurements (90 min), the 96-well plates were placed into a CO₂ incubator, and the infection was left to proceed for an additional 15 h. The cells were then fixed and stained as described above. The amount of virus that entered at each time point was compared to the amount of virus that entered when no EGTA wash was performed, which was taken as 100% entry.

Treatment of MA104 cells with inhibitors. Confluent monolayers of MA104 cells grown in 96-well plates were washed twice with MEM and were pretreated or not with different concentrations (indicated in each figure) of M β CD and bafilomycin A1 (for 1 h), NH₄Cl and DMA (for 30 min), or sucrose (for 10 min); all reagents were dissolved in MEM. After this incubation, cells were infected with the different rotavirus strains or with reovirus (2,000 FFU per well) for 1 h, with the different reagents (except M β CD) maintained during this period. Cells were washed twice with 3 mM EGTA in PBS to remove viral particles still present on the cell surface. Reovirus-infected cells were further incubated with an antireovirus polyclonal antibody for 15 min to neutralize the virus that remained on the cell surface. Then, the infection was left to proceed for an additional 15 h. For cholesterol replenishment assays, cells were washed twice with MEM and treated for 1 h with M β CD 10 mM. After this treatment, the cells were washed twice with MEM, and water-soluble cholesterol (100 μ g/ml) was added for 1 h. The cells were washed again and were infected with rotavirus strains for 1 h. Finally, the cells were washed and the infection was left to proceed for 15 h; all incubations were performed at 37°C. After incubation, cells were fixed, stained, and analyzed as described above. None of these treatments caused cell death as determined by morphological examination and trypan blue exclusion assays (results not shown).

Dextran uptake. Confluent monolayers of MA104 cells grown on coverslips in 48-well plates were washed twice with MEM and treated with DMA for 30 min. Then, the cells were cooled for 10 min at 4°C. After this period, the cells were incubated with TMR-dextran (100 μ M) in the presence of DMA for 30 min at 4°C. Prematured MEM was added, and the cells were shifted to 37°C for 45 min. Finally, dextran uptake was stopped by washing with cold MEM and the cells were fixed and prepared for immunofluorescence as described below.

Blocking of rotavirus infectivity with monoclonal antibodies to hsc70. Confluent monolayers of MA104 cells grown in 96-well plates were washed twice with MEM, and different concentrations of MAbs to hsc70 (MA3-006) or to hsp70 (MA3-009) were added to cells for 90 min at 37°C (18). After this incubation period, MAbs were removed and cells were infected with the different rotavirus strains (2,000 FFU per well) for 1 h at 37°C. After the adsorption period, the virus inoculum was removed, cells were washed twice with MEM, and the

infection was left to proceed for 15 h at 37°C. Infected cells were fixed and immunostained as mentioned above.

Plasmids and transfections. Plasmids pCDNA3.1/Dyn2 (for wild-type [wt] dynamin), which expresses N-terminally HA-epitope-tagged dynamin, and pCDNA3.1/K44A (for mutant dynamin), expressing an N-terminally HA-epitope-tagged K44A mutant, were kindly provided by S. L. Schmid (Scripps Research Institute, La Jolla, CA). pCINeo/IRES-GFP/caveolin-1 and pCINeo/IRES-GFP/caveolin-1 DN, which are bicistronic expression vectors expressing green fluorescent protein (GFP) and wild-type caveolin-1 or a mutant caveolin-1 from which residues 1 to 81 were deleted, respectively, were kindly donated by J. Eggermont, Katholieke Universiteit, Leuven, Belgium. Plasmids were transfected into 80% confluent cell monolayers grown on coverslips using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 48 h, cells were infected with the different virus strains as described above. The expression of wt dynamin and the K44A mutant was monitored by using a MAb against the HA tag, whereas the expression of wt caveolin and its mutant was monitored through GFP expression.

Transfection of siRNAs. Clathrin heavy chain small interfering RNA (siRNA) was obtained from Dharmacon Research (Lafayette, CO); the sequence of this siRNA was previously reported (2, 38). The siRNA used to knock down the expression of dynamin-2 was kindly provided by P. Cossart (Pasteur Institute, Paris, France) (57). As an irrelevant control, an siRNA to green fluorescent protein was used. Transfection of siRNAs into MA104 cells was performed using a reverse transfection method. Briefly, 15 μ l Oligofectamine (Invitrogen, Carlsbad, CA) was diluted in 1 ml MEM and incubated for 10 min at room temperature. This mixture was then added to a well of a 48-well plate containing the siRNA, also diluted in MEM. After an incubation of 20 min at room temperature, 200 μ l of a single-cell suspension of 0.5×10^5 MA104 cells/ml was added to each well, and the cells were incubated at 37°C. Seventy-two hours later, the transfection mixture was removed and the cells were washed twice with MEM and infected with rotavirus.

Lipofection of DLPs. siRNA-transfected cells were transfected with DLPs from different rotavirus strains using Lipofectamine (lipofection). Briefly, DLPs were diluted in MEM and incubated with a mixture of Lipofectamine (Invitrogen Carlsbad, CA) in MEM for 20 min at room temperature. One hundred microliters of this mixture was added to the cells for 1 h at 37°C, and then cells were washed with MEM. At 15 h postlipofection, cells were fixed, and infected cells were detected by a peroxidase focus-forming assay as described above.

Immunofluorescence (IF) assay. MA104 cells grown on glass coverslips to approximately 80% confluence were transfected with plasmids for transient expression assays or with siRNAs as described above, and at 48 h posttransfection (for plasmids) or 72 h posttransfection (for siRNAs), cells were infected with rotavirus or reovirus for 2 h at 37°C. Six hours postinfection (h.p.i.), the cells were fixed with 2% paraformaldehyde in PBS for 20 min. After this time, the cells were washed three times with PBS containing 50 mM NH_4Cl , permeabilized by incubation with 0.5% Triton X-100 in blocking buffer (50 mM NH_4Cl , 1% bovine serum albumin [BSA] in PBS) for 15 min, washed three times with PBS containing 50 mM NH_4Cl , and blocked by incubation with 1% BSA, 50 mM NH_4Cl in PBS at 4°C overnight. The coverslips were incubated with primary antibodies for 1 h, followed by incubation with the corresponding Alexa-labeled secondary antibodies for 1 h. All incubations were performed at room temperature. Coverslips were mounted on glass slides using Fluoromount (Argene, Varilhes, France), and slides were analyzed with a fluorescence microscope (Zeiss Axioskop 2 mot plus) coupled to a digital camera (Photometrics Cool Snap HQ). The images were then digitally captured and prepared in Adobe Photoshop 6.0.

Immunoblot analysis. Cells were lysed in Laemmli sample buffer, denatured by boiling for 5 min, subjected to SDS-PAGE, and transferred to Immobilon NC (Millipore) membranes. Membranes were blocked with PBS containing 5% nonfat dry milk and incubated with the antibodies indicated in Fig. 7 as previously described (36). Bound antibodies were developed by incubation with a peroxidase-labeled secondary antibody and the Western Lightning system (PerkinElmer).

Image analysis and statistical analysis. For quantification, nonmanipulated raw images were assessed and counted for infected/transfected cells. In all instances, data were acquired from at least three independent experiments. All statistical evaluations were carried out with the two-tailed paired *t* test using Prism 5.0.

RESULTS

Kinetics of entry of different rotavirus strains. To determine if receptor usage had some influence in the mechanism of

FIG. 1. Kinetics of rotavirus entry. MA104 cells grown in 96-well plates were infected with 2,000 FFUs of the indicated virus strains per well. Viral particles present at the cell surface were removed with 3 mM EGTA at the indicated time points, and the infection was allowed to proceed for 15 h at 37°C. Finally, cells were fixed and immunostained as described in Materials and Methods. Data are expressed as the percentage of the virus infectivity obtained in wells where viral particles of each strain were not removed with EGTA, which was taken as 100% infectivity. The arithmetic means and standard deviations of the results of three independent experiments performed in duplicate are shown.

rotavirus entry, we selected four rotavirus strains that have been shown to differ in their susceptibility to NA treatment of target cells and in their integrin dependence. Table 1 shows the four strains chosen for this study. As mentioned above, the interactions of rotavirus with the cell surface, as well as some aspects of the mechanism of entry, have already been characterized for the simian rotavirus RRV, which is NA sensitive and integrin dependent (5, 6, 17, 28, 33). Initially, we determined whether all four strains entered the cell at the same rate. For this, the different viruses were added to MA104 cell monolayers and allowed to bind for 1 h at 4°C; under these conditions, the viral particles bind to the cell surface but do not enter. Unbound viruses were washed off, and a synchronized entry process was started by adding warm medium to the cells. The entry of the viral particles was stopped at different times by removing the virus that had not entered cells with an EGTA wash, which releases the outer layer of the virions and causes the particles to detach from the cell surface. After this step, cells were incubated for 15 h and virus-infected cells were quantitated with an immunoperoxidase focus-forming assay. Figure 1 shows the results of these assays. The rate of entry of all four rotavirus strains differed greatly; RRV entered the cells with a half-time of about 35 min, whereas only 20% of strain UK had entered after 90 min of incubation. Strains TFR-41 and Wa had intermediate rates of entry, with half-times of approximately 60 and 90 min, respectively.

The infectivity of all four rotavirus strains depends on the presence of cholesterol. It has been shown that depletion of cholesterol from the cell membrane, using chelating agents such as methyl- β -cyclodextrin (M β CD), results in a severe decrease in the infectivity of the simian strain RRV, its variant nar3, and the human strain Wa (19). To determine if rotavirus strains TFR-41 and UK also depend on cholesterol for cell entry, the cells were treated with M β CD and the effect on the

FIG. 2. Cholesterol depletion reduces the infectivity of all four rotavirus strains. MA104 cells grown in 96-well plates were pretreated with 10 mM MβCD for 1 h at 37°C and washed with MEM, and then, water-soluble cholesterol (100 μg/ml) was added or not added to cells for 1 h at 37°C. Finally, all wells were infected with 2,000 FFUs of the indicated rotavirus strains. Fifteen h.p.i., infected cells were detected by an immunoperoxidase focus detection assay using a rabbit serum to rotavirus as indicated in Materials and Methods. Data are expressed as the percentage of the number of FFUs observed in control untreated cells, which was taken as 100% infectivity. The arithmetic means ± standard deviations of the results of four independent experiments performed in duplicate are shown.

infectivity of the viral strains was measured. The results in Fig. 2 show that all four rotavirus strains were susceptible to cholesterol depletion, suggesting that they all depend on the presence of cholesterol to infect the cells. To discard possible secondary effects associated with MβCD treatment, cholesterol was restored to cells after treatment with this drug and the infectivity was measured (Fig. 2). We found that cholesterol replenishment restored the infectivity of all rotavirus strains, suggesting that the presence of cholesterol is required for virus entry.

All rotavirus strains interact with hsc70. We have previously found that the heat shock protein hsc70 plays an important role as a postattachment receptor for RRV (18, 60). Here, we tested whether the other strains analyzed also used hsc70 during their interaction with the cell surface. In these assays, the cell monolayer was incubated with different concentrations of monoclonal antibodies directed to hsc70 or to hsp70 as a control. After this incubation, cells were infected with the different rotavirus strains and their infectivity was determined at 15 h.p.i. We found that the infectivity of Wa, TFR-41, and UK decreased by about 30 to 40% (depending on the viral strain) when the infection was performed in cells preincubated with the anti-hsc70 antibody, while the infectivity in cells that were preincubated with the control antibody to hsp70 was not affected (Fig. 3A and B). These results indicate that all four strains tested require the presence of hsc70 on the cell surface.

Preventing endosomal acidification affects the infectivity of some rotavirus strains. We and others (1, 25, 50) have found that preventing the acidification of endosomes does not affect the cell entry of RRV. Here, we tested the effects of NH₄Cl and bafilomycin A1 on the infectivity of Wa, TFR-41, and UK. Cells were preincubated with these reagents and were then infected with the different rotavirus strains. The effect of preventing the acidification of endosomes on the infectivity was determined by the detection of infected cells as described in Materials and Methods at 15 h.p.i. (Fig. 4). As expected, the

FIG. 3. Rotavirus infectivity is blocked by an antibody to hsc70. MAbs to hsc70 (A) or to hsp70 (B) were added to monolayers of MA104 cells for 90 min at 37°C. After this incubation period, cells were infected with the indicated rotavirus strains (2,000 FFUs per well) for 1 h at 37°C. After the adsorption period, the virus inoculum was removed and the infection was left to proceed for 15 h at 37°C. Infected cells were fixed and immunostained as indicated in Materials and Methods. Data are expressed as the percentage of the virus infectivity when cells were preincubated with PBS as control. The arithmetic means ± standard deviations of the results from at least two independent experiments are shown.

infectivity of RRV was not reduced by any of the concentrations of NH₄Cl tested. In contrast, the infectivity of the other rotavirus strains was affected, as was the infectivity of reovirus that was used as a positive control. Similar results were obtained with bafilomycin A1, which prevents acidification of the endosomes by inhibiting the vacuolar ATPase (12). The infectivity of RRV with this treatment was slightly but statistically significantly affected (Fig. 4). Taken together, these data suggest that strains TFR-41, Wa, and UK depend on the acidification of the endosome to efficiently infect MA104 cells, while RRV seems to be less sensitive to changes in the endosomal pH.

The low endosomal pH might be required to trigger conformational changes in some of the viral proteins needed for the virus to escape the endosomal compartment. Alternatively, an acidic pH might be required for optimal function of the endosomal cysteine proteases cathepsin L and cathepsin B, whose activity has been shown to be required for reovirus infectivity (14). To determine if cathepsin L was needed for rotavirus entry, we tested the effect of an inhibitor of this protease on the infectivity of the different rotavirus strains. Reovirus was used as a positive control for these experiments. We found that while the inhibition of cathepsin L severely blocked the infectivity of reovirus at the concentration tested, it did not signif-

FIG. 4. Effects on rotavirus infectivity of treatments that prevent the acidification of endosomes. MA104 cells grown in 96-well plates were pretreated with 100 mM NH_4Cl or with 0.5 μM bafilomycin A1 at 37°C for 30 min or 1 h, respectively. Cells were then infected with 2,000 FFUs of the indicated rotavirus strains, maintaining the reagents during the adsorption period of 1 h at 37°C. The virus inoculum was removed, and the infection was left to proceed for 15 h at 37°C. Infected cells were fixed and immunostained as indicated in Materials and Methods. Data are expressed as the percentage of the virus infectivity observed in control untreated cells, taken as 100%. The arithmetic means $\pm$ standard deviations of the results of at least five independent experiments performed in duplicate are shown. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ (paired t test).

icantly affect the infectivity of any rotavirus strain (data not shown).

Macropinocytosis and caveola-mediated endocytosis are not involved in rotavirus entry. To test whether some rotavirus strains enter cells through macropinocytosis or caveola-mediated endocytosis, we inhibited these two pathways and determined the effect on rotavirus infection. To inhibit the caveolin-dependent route, we transfected the cells with a construct expressing a dominant-negative mutant form of caveolin-1 (with amino acids 1 to 81 deleted) or with a plasmid encoding the wild-type form of this protein (54). The results in Fig. 5A show that cells expressing the dominant-negative mutant caveolin-1 were infected by all four rotavirus strains but were not infected by SV40, which has been previously shown to enter through caveola-mediated endocytosis and which was used as a positive control in these assays.

To block macropinocytosis, we treated the cells with 5-(N,N -dimethyl)amiloride hydrochloride (DMA), an inhibitor of the Na^+/H^+ pump, which has been shown to inhibit this pathway (31). None of the rotavirus strains tested were affected by this treatment (Fig. 5B), whereas the uptake of fluorescently labeled dextran, which is known to enter through macropinocytosis, was inhibited (data not shown). Together, these results suggest that neither macropinocytosis nor caveola-mediated endocytosis is involved in rotavirus entry.

Treatments that inhibit clathrin-mediated endocytosis alter the infectivity of some rotavirus strains. To establish if clathrin-dependent endocytosis is involved in the entry of the strains studied, we determined the effect of treating MA104 cells with a hypertonic medium (containing sucrose at different concentrations), which results in the dissociation of clathrin vesicles from the plasma membrane (20, 21). The results in Fig. 6A show that, while the infectivity of RRV was barely affected at 250 mM, the highest sucrose concentration tested, the infectivity of the other three strains and reovirus decreased signif-

FIG. 5. Blocking caveola-mediated endocytosis or macropinocytosis does not reduce rotavirus infectivity. (A) MA104 cells were transfected with plasmids expressing wild-type or the dominant-negative mutant of caveolin-1. Transfected cells were infected with the different viruses and processed for IF assay. Both wild-type and mutant proteins were detected through GFP expression. Infected cells were fixed and immunostained as indicated in Materials and Methods. The numbers of infected cells in the GFP-positive transfected cells were scored ($n \geq 100$), and infectivities are expressed as percentages of the infected cell number in the cells transfected with wild-type or dominant-negative constructs. The arithmetic means $\pm$ standard deviations of the results of at least three independent experiments are shown. (B) MA104 cells grown in 96-well plates were pretreated with the indicated concentrations of DMA at 37°C for 30 min. Cells were then infected with 2,000 FFUs of the indicated rotavirus strain, maintaining DMA during the adsorption period of 1 h at 37°C. The virus inoculum was removed, and the infection was left to proceed for 15 h at 37°C. Infected cells were fixed and immunostained as indicated in Materials and Methods. Data are expressed as the percentages of the virus infectivity observed in control untreated cells, representing 100%. The arithmetic means $\pm$ standard deviations of the results of at least three independent experiments performed in duplicate are shown. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ (paired t test).

icantly. These results point out the fact that different rotavirus strains may enter the cells through different pathways.

To confirm the role of the clathrin-mediated endocytosis pathway in the entry of some rotavirus strains, we silenced the expression of the clathrin heavy chain by RNA interference (RNAi), treatment that blocks the formation of clathrin cages, and in consequence, the uptake of clathrin-mediated endocytosis ligands, such as transferrin (38). Cells were transfected with an siRNA directed to the clathrin heavy chain, and at 72 h posttransfection, the cells were infected with different rotavirus strains. At 6 h.p.i., cells were fixed and stained with antibodies to clathrin and to rotavirus, and the number of infected cells that were transfected with the siRNA against clathrin were counted and compared with the number of infected cells that were detected when an irrelevant siRNA was used as a control.

FIG. 6. Effects on rotavirus infectivity of treatments that affect clathrin-mediated endocytosis. (A) MA104 cells grown in 96-well plates were pretreated with the indicated concentrations of sucrose for 10 min at 37°C. Cells were then infected with 2,000 FFUs of the indicated rotavirus strains, maintaining the hypertonic medium during the adsorption period of 1 h at 37°C. The virus inoculum was removed, and the infection was left to proceed for 15 h at 37°C. Infected cells were fixed and immunostained as indicated in Materials and Methods. Data are expressed as the percentage of the virus infectivity observed in control untreated cells, which represents 100%. The arithmetic means $\pm$ standard deviations of the results of five independent experiments performed in duplicate are shown. (B) Immunofluorescence assay showing the silencing of clathrin heavy chain. MA104 cells grown in coverslips were transfected with the indicated siRNAs (Irre, irrelevant GFP siRNA); at 72 h posttransfection, the cells were fixed and processed for IF using an antibody to the clathrin heavy chain (green), and the nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI; blue). (C) MA104 cells grown in coverslips were transfected with the indicated siRNAs and, at 72 h posttransfection, were infected with the different strains of rotavirus or reovirus. At 6 h.p.i., cells were fixed and processed for IF as indicated in Materials and Methods. Rotavirus-infected cells were monitored using an anti-NSP2 rabbit antibody, and reovirus-infected cells were detected by an anti-reovirus rabbit antibody, followed by incubation with antirabbit Alexa 568. The numbers of infected cells were scored, and the infectivities are expressed as percentages of the numbers of infected cells found in the cells transfected with the siRNAs ($n \geq 150$). The arithmetic means $\pm$ standard deviations of the results of at least three independent experiments are shown. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ (paired t test).

The results in Fig. 6B show that there was a very efficient knockdown of the expression of clathrin, since few cells (approximately 10% of the control-transfected cells) were still able to express this protein. Under these conditions, the uptake of fluorescently labeled transferrin was blocked (data not shown). When the effect on infectivity of silencing this protein was measured, we found that the infectivity of RRV was not significantly affected by this treatment, confirming our previous results (50). In contrast, the infectivity of TFR-41, UK, Wa, and reovirus was decreased when the clathrin heavy chain was knocked down. The human strain Wa appeared to be the most sensitive, since there was about an 80% reduction of its infectivity under these conditions (Fig. 6C).

Role of dynamin in rotavirus entry. Dynamin is a large GTPase that is responsible, among other things, for scission of vesicles from the plasma membrane (10). A dominant-negative mutant form of dynamin II, mutant K44A that contains a single amino acid change in the GTPase domain, has been used extensively to inhibit both caveolar and clathrin endocytosis and to define the role of these pathways in the cell entry of several viruses (16, 24, 44, 58). In order to define the role of dynamin in the entry of rotaviruses, MA104 cells were transiently transfected with plasmids that encode either Dyn-wt or the mutant Dyn-K44A, as described in Materials and Methods. The transfected cells were then infected with the different strains of rotavirus or with reovirus, and the number of transfected cells (as detected by an anti-HA antibody [see Materials and Methods]) that were infected was scored (Fig. 7A). We found that the infectivity of all the strains tested was decreased by about 70%, suggesting that dynamin is involved in the entry of all rotavirus strains tested.

Dynamin and clathrin are important during the entry step of rotaviruses. Since it was conceivable that the knockdown of either clathrin or dynamin affected the infectivity of rotaviruses at a postentry step (39, 48), we analyzed whether the inhibitory role of these two proteins was during the entry process. For this, the expression of dynamin and clathrin was silenced by RNAi and cells were transfected with purified DLPs from RRV, TFR-41, and UK viruses. DLPs lack the outer layer proteins and, thus, are not able to bind and enter MA104 cells. Transfection of DLPs into cells overcomes the entry step of the virions. If the absence of clathrin or dynamin were affecting a process after viral entry, we would expect the infectivity of the transfected DLPs to be decreased. We found that silencing the expression of clathrin or dynamin did not affect the infectivity of any of the viral strains tested (Fig. 7B), suggesting that the effect of their absence is indeed at the virus entry level. The knockdown effectiveness of each of these proteins was assessed by Western blot analysis (Fig. 7C). The protein level of the clathrin heavy chain was reduced almost 100%, whereas there was approximately 11% dynamin II still expressed in siRNA-transfected cells. We were not able to purify enough viral particles from the human strain Wa to perform these assays, but they would be expected to behave like the other rotavirus strains.

DISCUSSION

The early interactions of rotavirus with its host cell have been extensively characterized for simian strain RRV (19, 50,

FIG. 7. Rotavirus entry depends on clathrin and dynamin II. (A) MA104 cells were transfected with plasmids expressing the wild type or the K44A form of dynamin II. Transfected cells were infected with the different viruses and processed for IF. Both wild-type and K44A proteins were detected with an anti-HA tag MAb. Infected cells were fixed and immunostained as indicated in Materials and Methods. The numbers of infected cells in the HA-positive transfected cells were scored ($n \geq 100$), and the infectivities are expressed as the percentages of the infected cell number in the cells transfected with wild-type or dominant-negative constructs. The arithmetic means $\pm$ standard deviations of the results of at least three independent experiments are shown. REO, reovirus. (B) siRNA-transfected cells were transfected with DLPs of the indicated rotavirus strains for 1 h at 37°C. After this period, the transfection mixture was removed and cells were kept in MEM. At 15 h.p.i., cells were fixed and immunostained as indicated in Materials and Methods. The data are expressed as the percentage of the number of FFUs observed in the control (irrelevant siRNA-transfected cells), which represents 100%. The arithmetic means $\pm$ standard deviations of the results of at least three independent experiments performed in duplicate are shown. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ (paired t test). (C) Immunoblot analysis of cell lysates from cells transfected with the indicated siRNA (Irre, irrelevant GFP siRNA). The antibodies used are indicated as follows: α -Clathrin, anti-clathrin heavy chain; α -Dyn2, antidyminin; α -Vim, antivimentin (used as a loading control).

60); however, differences among rotavirus strains in their use of cellular receptors (5, 6, 17, 34) leave open the question of whether they all enter the cell through the same pathway. We chose four rotavirus strains that differ in their requirements of interaction with sialic acids and integrins. RRV was used as a strain representative of the NA-sensitive, integrin-dependent phenotype and for comparison with the other strains selected (Table 1).

We initially evaluated the entry rates of the four selected strains and found that each strain entered the cell at a different

rate (Fig. 1). Despite these differences, all four strains depended on the presence of cholesterol on the cell surface. Interestingly, the only interaction that all strains had in common with the previously characterized rotavirus receptors was with the heat shock cognate protein hsc70, since antibodies to this protein blocked the infectivity of all four strains at very similar levels.

When the effect on viral infectivity of preventing the acidification of endosomes was determined, we found that the entry of strains TFR-41, Wa, and UK was pH dependent, while RRV did not seem to require a low endosomal pH, although a slight but significant effect of bafilomycin A was observed for this strain. The inhibition of cathepsin L did not affect the infectivity of any of the four rotavirus strains tested (results not shown), suggesting that the dependence of TFR-41, Wa, and UK on endosomal acidification is not due to a requirement of the activation of this protease. Since viruses internalized through clathrin-mediated endocytosis usually require an acidic environment (42), we evaluated the role of this endocytic pathway in the entry of the different strains. To our surprise, and in contrast to our previous findings with RRV, we found that treatment of cells with a hypertonic medium, which precludes the formation of clathrin cages, and silencing the expression of the clathrin heavy chain, which is an essential component of the clathrin triskelion (15, 59), decreased the infectivity of all the strains except RRV, suggesting that the entry of these viruses is through a clathrin-mediated endocytosis. Accordingly, we also found that all viruses tested depended on the presence of a functional dynamin.

The use of reovirus type 1 as a positive control for many of the assays performed in this work demonstrated that this virus enters MA104 cells through clathrin-mediated endocytosis, as has been described in L cells (30). This observation is interesting in light of recent findings that have established that the entry route of some viruses depends not only on the virus but also on the cell line tested. For example, SV40, a virus whose entry has been extensively studied, behaved differently in CV1 cells, where the virus enters through caveolae, and in Huh-7 cells, where the virus does not use this entry pathway (9, 43). As has been shown previously (50) and in this study, SV40 enters MA104 cells through a caveolin-dependent pathway.

The results presented in this work show that different rotavirus strains differ not only in their receptor usage but also in their entry pathway. Table 2 summarizes the results found in

TABLE 2. Summary of the results obtained in this work

Treatment	Effect on infectivity of strain:			
	RRV	TFR-41	UK	Wa
MβCD	***	***	***	***
Hsc70	**	**	**	**
NH ₄ Cl	—	***	***	***
Bafilomycin A1	*	**	***	**
Sucrose	—	**	***	***
Clathrin	—	***	***	***
Caveolae	—	—	—	—
Macropinocytosis	—	—	—	—
Dynamin	***	***	***	***

—, no effect; *, <30% reduction; **, 30 to 50% reduction; ***, >50% reduction.

this study. Interestingly, a correlation between the cell receptors used and their entry pathway was not found. RRV, the virus that seems to enter cells faster, is the only virus that does not enter the cell via clathrin- or caveola-mediated endocytosis (50; this work) or macropinocytosis (this work). The other three virus strains tested depend on clathrin-mediated endocytosis (caveolin-dependent or macropinocytosis uptake were ruled out).

The findings in this work could help to clarify the different, sometimes even contradictory results which have been reported in the literature when characterizing different rotavirus strains (22). For instance, different results have been reported for the role of low pH on entry and for the role of the Ca^{2+} concentration necessary for viral uncoating. It was reported that the infectivities of porcine strain OSU and simian strain SA11 were affected when the cells were treated with bafilomycin A1, an inhibitor of the endosomal H^+ -ATPase (12), whereas under the same conditions, the infectivity of RRV was not affected (3, 8). Similar results were obtained when compounds that raise the intracellular calcium concentration were used with different rotavirus strains (8, 29). These apparently contradictory results might be explained by intrinsic differences in the rotavirus strains used in each work, and most probably not by the different experimental conditions employed. It is also important to point out that the rotavirus strains that have been used in these studies have been adapted to grow in cell culture for a long time, and their entry modes may differ from those of wild-type strains when infecting enterocytes.

It has already been observed that viruses belonging to the same genus use different endocytosis pathways to infect the host cell. Human rhinovirus 14 (HRV14) and HRV2 use different receptors for host cell attachment and, similar to rotavirus strains, the uptake of these rhinoviruses by the cells is different; HRV2 enters through the clathrin-mediated pathway, whereas HRV14 entry is independent of clathrin, caveolin, and flotillin (26, 51). This phenomenon is also illustrated among the *Polyomaviridae* family; human polyomaviruses JCV and BKV enter the cell through clathrin-dependent and caveola-mediated endocytosis, respectively (13, 37, 45–47), although they finally converge in the endoplasmic reticulum (47). The observation that rotaviruses isolated from different animal origins use distinct entry mechanisms is thus not unexpected. It is tempting to speculate that rotavirus affinity, the order of interactions, or the signaling molecules activated through virus binding to its receptors may induce a particular endocytic mechanism. It remains to be determined whether or not the vesicular trafficking used by all these viruses converges at some point such that they share their final destination in the cell's cytoplasm to start transcription at the same cellular site.

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