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Heparan Sulfate-Binding Foot-and-Mouth Disease Virus Enters Cells via Caveola-Mediated Endocytosis[▽]

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Foot-and-mouth disease virus (FMDV) utilizes different cell surface macromolecules to facilitate infection of cultured cells. Virus, which is virulent for susceptible animals, infects cells via four members of the α_V subclass of cellular integrins. In contrast, tissue culture adaptation of some FMDV serotypes results in the loss of viral virulence in the animal, accompanied by the loss of virus' ability to use integrins as receptors. These avirulent viral variants acquire positively charged amino acids on surface-exposed structural proteins, resulting in the utilization of cell surface heparan sulfate (HS) molecules as receptors. We have recently shown that FMDV serotypes utilizing integrin receptors enter cells via a clathrin-mediated mechanism into early endosomes. Acidification within the endosome results in a breakdown of the viral capsid, releasing the RNA, which enters the cytoplasm by a still undefined mechanism. Since there is evidence that HS internalizes bound ligands via a caveola-mediated mechanism, it was of interest to analyze the entry of FMDV by cell-surface HS. Using a genetically engineered variant of type O_1Campos (O_1C3056R) which can utilize both integrins and HS as receptors and a second variant (O_1C3056R-KGE) which can utilize only HS as a receptor, we followed viral entry using confocal microscopy. After virus bound to cells at 4°C, followed by a temperature shift to 37°C, type O_1C3056R-KGE colocalized with caveolin-1, while O_1C3056R colocalized with both clathrin and caveolin-1. Compounds which either disrupt or inhibit the formation of lipid rafts inhibited the replication of O_1C3056R-KGE. Furthermore, a caveolin-1 knockdown by RNA interference also considerably reduced the efficiency of O_1C3056R-KGE infection. These results indicate that HS-binding FMDV enters the cells via the caveola-mediated endocytosis pathway and that caveolae can associate and traffic with endosomes. In addition, these results further suggest that the route of FMDV entry into cells is a function solely of the viral receptor.

Foot-and-mouth disease virus (FMDV) is the type species of the genus *Aphthovirus* of the family *Picornaviridae*. The virus is the cause of a highly contagious disease of cloven-hoofed livestock, which is characterized by vesicular lesions in the mouth and on the feet, teats, and nares (see reference 31 and references therein). It has been well established that, via a conserved Arg-Gly-Asp (RGD) sequence in the βG-to-βH (G-H) loop of VP1 (6, 26, 41, 49), the virus utilizes four members of the α_V subgroup of cellular integrins ($\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_6$, and $\alpha_V\beta_8$) as receptors in tissue culture (12, 21, 34, 37, 38, 54).

In addition to cellular integrins, FMDV is also able to utilize alternative membrane molecules as receptors. The first evidence of this was presented in reports that found antibody-complexed virus was able to bind to Fc receptors and that this binding led to internalization and viral replication (7, 47, 49). This work was expanded to develop an artificial receptor by fusing a single-chain monoclonal antibody against FMDV (46) to the intercellular adhesion molecule 1 (ICAM-1), engineering a receptor designed to allow virus with an altered receptor binding site with which to attach and replicate in cells (67). Finally, it was demonstrated that some FMDV serotypes, when adapted to tissue culture, gain the ability to utilize cell surface heparan sulfate (HS) as a viral receptor (2, 35, 45, 54), resulting in the attenuation of virulence in host species (70). The interactions between these virions and HS have recently been delineated by crystallographic analyses (19, 27–29). These data, along with recent evidence that FMDV interaction with soluble $\alpha_V\beta_6$ integrin does not result in structural alterations to the virion (22), suggest that the viral receptor plays a role only in docking and internalizing the virion. However, in spite of the ability of the virion to utilize alternative receptors, the available evidence suggests that the integrin receptor is the only one involved in viral pathogenesis in the susceptible host (52, 54, 68; V. O'Donnell, A. Koser, D. Gregg, and B. Baxt. Abstr. XIIIth Meeting of the European Study Group for the Molecular Biology of Picornaviruses. Lunteren, The Netherlands, 23 to 29 May 2005).

Recently, studies of the entry of FMDV into cultured cells have been initiated. Results from both our laboratory and that of Jackson and colleagues have demonstrated that integrin-binding FMDV types A and O utilize a clathrin-dependent mechanism to infect cells, trafficking through early endosomes into recycling endosomes (13, 58). It is within these endosomes that the virion breaks down (3–5, 16, 51), releasing the viral genome to the cytosol. More recently, Martin-Acebes et al. (44) also demonstrated the involvement of a clathrin-dependent mechanism in productive FMDV type C endocytosis. It was of interest to us to determine the mechanism of entry for FMDV when the virus uses a receptor other than an integrin, for example, HS. There is strong evidence that ligands that bind to HS on HS proteoglycans, including those of bacteria

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and viruses, are internalized via caveola-mediated endocytosis, although this route is dependent on the protein moiety of the HS proteoglycan (11, 23, 25, 65). A recent report, however, suggests that human papillomavirus binds to HS on dendritic cells and is internalized by a clathrin-dependent pathway (14).

In this study, we followed the internalization, via confocal microscopy, of an FMDV variant which can utilize either HS or integrins as the receptor. In addition, we utilized a genetically engineered variant of this virus which can utilize only HS as a receptor and compared variant entry events to those of the integrin-binding parent virus. Our results demonstrate that the entry mechanism of FMDV into cultured cells is dependent on the receptor to which the virus binds.

MATERIALS AND METHODS

Cell lines and viruses. Human mammary gland epithelial cells (MCF-10A) were obtained from ATCC (catalogue no. CRL-10317) and were maintained in a mixture of Dulbecco's minimum essential medium (DMEM) and Ham's F-12 medium (1:1; Invitrogen, CA) containing 5% heat-inactivated fetal bovine serum (FBS), 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 500 ng/ml hydrocortisone. COS-1 cells were maintained in DMEM containing 10% FBS, an additional 2 mM L-glutamine, and 1 mM sodium pyruvate.

The FMDV type O_1 strain Campos (O_1Campos) was derived from the vesicular fluid of an experimentally infected steer. The HS and integrin-binding variant, O_1C3056R, was derived from an O_1Campos vaccine seed stock by the insertion of the capsid-coding region of a small-plaque variant virus, selected on CHO cells, into an FMDV type A_{12} infectious cDNA clone (in reference 70, the virus was referred to as vCRM4). A variant of this virus, in which the RGD integrin recognition site in VP1 was mutated to a Lys-Gly-Glu (KGE) sequence by site-directed mutagenesis (O_1C3056R-KGE), was derived from the infectious cDNA clone pCRM48 (70).

Antibodies and reagents. Monoclonal antibody (MAb) 12FB directed against type O_1 was diluted 1/5 for use and has been previously described (77). The MAbs LM609 (catalogue no. MAB1976), which recognizes the $\alpha_V\beta_3$ heterodimer (17), and CSβ6 (catalogue no. MAB2076), which recognizes the β_6 integrin subunit (86) and the $\alpha_V\beta_6$ heterodimer (22), were purchased from Chemicon (Billerica, MA) and diluted 1/100 for use. To study virus entry, antibodies against the following human cellular structures were used. A rabbit polyclonal antibody against early endosomal antigen-1 (EEA-1; Affinity Bioreagents, Golden, CO) was used at a 1/100 dilution to identify early endosomes; early and recycling endosomes also were identified by using rabbit polyclonal antibodies against Rab4 (72, 76, 83) (StressGen, Ann Arbor, MI) and Rab11 (75, 76, 87) (Affinity BioReagents) at a 1/100 dilution; a rabbit polyclonal antibody against caveolin-1 and a MAb against clathrin heavy chain (Affinity Bioreagents), each at a 1/200 dilution, were used as markers for caveolae and clathrin endocytosis pathways, respectively; MAbs directed against the endoplasmic reticulum marker protein disulfide isomerase (PDI; clone RL77; 1/100 dilution; Affinity Bioreagents), the Golgi apparatus markers, the Golgi zone area (clone 371-4; 1/50 dilution; Chemicon), and the coatomer protein I (COPI; clone maD; 1/100 dilution; Sigma, St. Louis, MO), and transferrin receptor (TfnR; clone RVS-10; 1/50 dilution; Chemicon) were used.

The following chemicals and inhibitors were used. Chlorpromazine (Sigma), which causes the loss of coated pits from the surface of the cell and the appearance of clathrin coats, composed of the same subunits on endosomal membranes (39, 85), was used at a 12.5 μM concentration; cytochalasin D (Sigma), which disrupts the actin cytoskeleton (18) and inhibits lipid raft-mediated internalization (59), was used at a 2 μM concentration; nystatin (Invitrogen), which sequesters cholesterol and disrupts lipid rafts (1, 74), and progesterone (Sigma), which inhibits synthesis of cholesterol (50), were used at concentrations of 25 μg/ml and 10 μg/ml, respectively; amiloride (Sigma), which inhibits macropinocytosis, was used at a 10 μM concentration; brefeldin A (Sigma), which disrupts the Golgi apparatus and affects the budding of COPI-coated membranes (30), was used at a concentration of 5 μg/ml; nocodazole (Sigma), which causes the depolymerization of microtubules and blocks endosomal traffic between peripheral early and late endosomes (10, 32), was used at a concentration of 20 μM; monensin ionophore (Sigma) and ammonium chloride (Sigma), lysosomotropic agents which raise the pH in endocytic vesicles (43), were used at concentrations of 50 μM and 50 mM, respectively.

Viral growth curve. Monolayers of MCF-10A cells were infected with the type virus O_1Campos, O_1C3056R, or O_1C3056R-KGE at a multiplicity of infection (MOI) of 10 PFU/cell for 1 h at 37°C. At the end of the adsorption period, the inoculum was removed, and the cells were rinsed with ice-cold 2-morpholinoethanesulfonic acid (MES)-buffered saline (25 mM MES [pH 5.5], 145 mM NaCl) to inactivate residual virus particles. The monolayers were then rinsed with MEM (Invitrogen) containing 1% FBS and 25 mM HEPES (pH 7.4) and incubated at 37°C. At the appropriate times postinfection (p.i.), the cells were frozen at −70°C, and the thawed lysates were used to determine titers by plaque assay on BHK-21 cell monolayers.

Infection of cells and confocal microscopy. Subconfluent monolayers of MCF-10A cells grown on 12-mm glass coverslips in 24-well tissue culture dishes were infected with FMDV at an MOI ranging from 50 to 100 PFU/cell for 1 h at 4°C in MEM containing 0.5% FBS and 25 mM HEPES (pH 7.4). After the adsorption period, the inoculum was removed, the monolayers were washed with MEM (Invitrogen), fresh medium was added, and the cells were incubated at 37°C. To analyze the role of lipid rafts in the internalization, MCF-10A cells were infected with virus as described above in the presence of 1 μg/ml of cholera toxin subunit B conjugated with Alexa Fluor 488 (Molecular Probes, Invitrogen, CA). To study the role of early endosomes and recycling endosomes, we infected MCF-10A cells with virus, as described before, in the presence of 200 μg/ml of transferrin conjugated with Alexa Fluor 488 (Molecular Probes). At the appropriate times, cells were fixed with 4% paraformaldehyde (EMS, Hatfield, PA) and processed for immunofluorescence and confocal microscopy as previously described (58). Briefly, after fixation, the paraformaldehyde was removed, and the cells were permeabilized with 0.5% Triton X-100 for 5 min at room temperature (RT) and incubated in blocking buffer (phosphate-buffered saline [PBS], 5% normal goat serum, 2% bovine serum albumin, 10 mM glycine) for 1 h at RT. The fixed cells were then incubated with the primary antibodies overnight at 4°C. After cells were washed with PBS, they were incubated with the appropriate secondary antibody, goat anti-rabbit immunoglobulin G (IgG) (1/400 dilution; Alexa Fluor 594; Molecular Probes) or goat anti-mouse isotype-specific IgG (1/400 dilution; Alexa Fluor 488 or Alexa Fluor 594; Molecular Probes), for 1 h at RT. Following this incubation, the cells were washed with PBS, counterstained with the nuclear stain TOPRO-iodide 642/661 (Molecular Probes) for 5 min at RT, washed, mounted, and examined with a Leica scanning confocal microscope (model TCS2). Data were collected utilizing the appropriate prepared controls lacking the primary antibodies, as well as the anti-FMDV antibodies with uninfected cells to give the negative background levels and to determine channel crossover settings. The captured images were adjusted for contrast and brightness, using Adobe Photoshop software.

Viral replication in the presence of internalization inhibitors. MCF-10A cells were incubated with the compounds at the concentrations listed above for 30 min to 2 h at 37°C prior to infection. The cells were then infected with FMDV O_1Campos or O_1C3056R-KGE at an MOI of 10 PFU/cell in the presence of the compounds. At the end of the adsorption period, the virus was removed, and the cells were rinsed once with ice-cold MES-buffered saline to inactivate unadsorbed virus. The cells were washed once with medium, and then medium containing the inhibitors was added. One set of cultures was immediately frozen at −70°C, and a second set was incubated for an additional 4 h at 37°C and then removed to −70°C. The plates were thawed, cell debris was removed by centrifugation, and viral titer was determined by plaque assay with BHK-21 cell monolayers. The results are represented as the ratio between the titers at 5 h postinfection (p.i.) and those at 1 h p.i.

Knockdown of caveolin-1 by RNA interference. The short interfering RNA (siRNA) SMARTpool, consisting of four RNA duplexes designed to target the gene of interest, human caveolin-1 (catalog no. D-003467), and a control siRNA, siGlo RISC-free (catalog no. D-001600-01), were purchased from Dharmacon (Lafayette, CO). Pools consisted of equal amounts of each duplex. The siRNA sequences are given as sense/antisense pairs. The four siRNA sequences for caveolin-1 were CUAAACACCUCAACGUGAUU/5′-PUCAUCGUUGAGGUGUUUAGUU, GCAGUUGUACCAUGCAUUAUU/5′-PUAAUGCAUGGUACAACUGCUU, AUUAAGAGCUUCCUGAUUGUU/5′-PCAAUCAGGAAGCUCUUAAUUU, and GCAAAUACGUAGACUCGGAUU/5′-PUCCGAGUCUACGUAUUUGCUU.

MCF-10A cells were grown to densities of 1.1×10^5 cells per well in 24-well tissue culture dishes in 1 ml of medium without antibiotics and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For each 2-cm dish, 100 total pmol of pooled siRNA was diluted in 50 μl of serum-free OptiMEM (Invitrogen), and, separately, 5 μl of Lipofectamine 2000 was diluted in 50 μl of OptiMEM. After an incubation of 5 min at RT, the diluted RNA and the Lipofectamine 2000 were combined and incubated for 20 min at RT. MCF-10A medium was replaced with 400 μl of serum-

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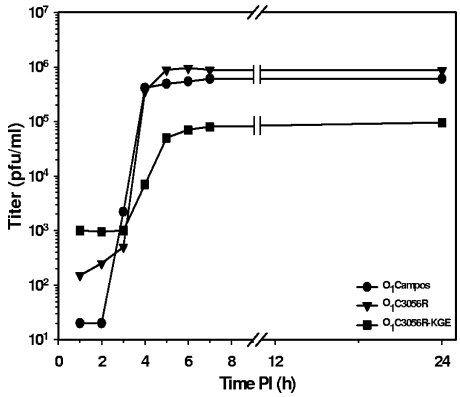

FIG. 1. Growth curve of FMDV type O_1 variants in MCF-10A cells. Cells were infected with FMDV type O_1Campos, O_1C3056R, or O_1C3056R-KGE at an MOI of 10 PFU/cell, as described in Materials and Methods. Combined intracellular and extracellular plaque titers were determined at different times p.i. in BHK-21 cells.

free OptiMEM, and the 100-μl mixture was then added to each dish, which was then rocked gently to spread the lipid-RNA complexes. Forty-eight hours after cells were transfected, they were infected with the O_1Campos or O_1C3056R-KGE virus at an MOI of 1 for 1 h at 37°C in MEM containing 0.5% FBS and 25 mM HEPES (pH 7.4). After the adsorption period, the inoculum was removed, and the cells were rinsed with ice-cold MES-buffered saline to inactivate residual virus particles. The monolayers were then rinsed with MEM containing 1% FBS and 25 mM HEPES (pH 7.4) and incubated at 37°C. One set of cultures was immediately frozen at −70°C, and a second set was incubated for an additional 4 h at 37°C and then removed to −70°C. The plates were thawed, cell debris was removed by centrifugation, and the viral titer was determined by plaque assay with BHK-21 cell monolayers. For analysis of caveolin-1 protein expression, Western blotting was performed 48 h after transfection. Total cell extracts were made using NET-1% Triton extraction buffer (150 mM NaCl, 1 mM EDTA,10 mM Tris [pH 7.5], 1% Triton X-100) supplemented with protease inhibitors (Roche, Mannheim, Germany). Extract was separated on a 15% Laemmli gel and transferred to a polyvinylidene difluoride membrane for Western blotting. Anti-caveolin-1 immunoblotting was performed using a rabbit anti-caveolin-1 antibody (Affinity Bioreagents), and anti-GAPDH immunoblotting was performed using a mouse anti-GAPDH antibody (Research Diagnostics). Antibodies were diluted 1/500 in a PBS solution containing 0.1% Tween-20 and 5% milk and detected with alkaline-phosphatase-conjugated goat anti-rabbit or anti-mouse antibody at a 1/1,000 dilution and 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP)/Nitro Blue Tetrazolium (NBT) (KPL, Baltimore, MD) as the substrate.

RESULTS

Growth kinetics of FMDV type O_1Campos variants in MCF-10A cells. We had previously shown that type O_1Campos replicates in MCF-10A cells via its interaction with the $\alpha_V\beta_6$ integrin (58) and that O_1C3056R was able to infect cells lacking the integrin receptor expression via its interaction with HS (54). To compare the growth of these two variants with that of type O_1C3056R-KGE, which is unable to bind to α_V integrin receptors, we analyzed the growth kinetics of these viruses in MCF-10A cells. The results shown in Fig. 1 demonstrate that O_1Campos and O_1C3056R both grew with similar kinetics and reached comparable titers in this cell line. Type O_1C3056R-KGE also grew in this cell line, although at a slower rate and to about 10-fold lower titers. These results indicated that MCF-10A cells are suitable for the study of the internalization events in vitro.

Heparin-binding type O_1Campos variants can bind and enter cells lacking integrin receptors. The $\alpha_V\beta_6$ integrin is present only on a subset of MCF-10A cells (58), so it was of interest to examine the early interactions between the O_1Campos variants and this cell type, which allowed us to analyze different entry mechanisms in the same cell line. Figure 2 shows the expression of the $\alpha_V\beta_6$ integrin and the binding and entry of each of the variants to MCF-10A cells. As we reported previously (58), upon adsorption, type O_1Campos bound only to cells expressing the $\alpha_V\beta_6$ integrin and colocalized with the integrin on the cell membrane at 4°C (Fig. 2a), and after the temperature was shifted to 37°C, the virus internalized in association with the integrin (Fig. 2b to d). In contrast, type O_1C3056R-KGE bound to cells regardless of whether the integrin was expressed or not, but in cells in which it was expressed, the virus and integrin did not colocalize (Fig. 2). This could be seen more dramatically when the temperature was shifted to 37°C and the entry of the virus followed (Fig. 2f to h). The binding of virus to the cell surface was abolished when the cells were treated with heparinase III (more than 70% inhibition; not shown). Type O_1C3056R, which contains the RGD sequence in VP1 and can utilize both integrins and HS as the receptors (54), also bound to cells regardless of $\alpha_V\beta_6$ expression (Fig. 2i). Unlike the variant with the KGE sequence in VP1, the type O_1C3056R virus colocalized with the integrin in cells expressing the receptor (Fig. 2j to l).

Integrin-binding and HS-binding FMDVs enter cells via different mechanisms. Since integrin-binding FMDVs enter cells via clathrin-mediated endocytosis (13, 58), it was of interest to examine HS-binding viruses to determine whether they utilized the same route of entry. Virus was allowed to adsorb to cells at 4°C for 1 h, the temperature shifted to 37°C, and the cells were fixed at different times after the temperature shift. Cells were probed for virus and either clathrin or caveolin-1 as described in Materials and Methods (Fig. 3), 15 min after the temperature shift. Type O_1Campos colocalized with clathrin (Fig. 3c); however, type O_1C3056R-KGE did not colocalize with clathrin (Fig. 3i), and type O_1C3056R colocalized with clathrin in some cells (Fig. 3o). When we probed with an antibody to caveolin-1, we observed that type O_1Campos did not colocalize with this protein (Fig. 3f), while type O_1C3056R-KGE did (Fig. 3l), and type O_1C3056R colocalized in some cells (Fig. 3r). Thus, virus which utilized HS as the receptor appeared to enter the cells via a different route than virus which bound to the $\alpha_V\beta_6$ receptor.

HS-binding FMDV moves through the endocytic pathway at a rate that is slower than integrin-binding virus. We, and others, had previously reported that integrin-binding FMDVs move rapidly through early endosomes into recycling endosomes (13, 58). Our results were based on the rapid colocalization of the incoming virus with the EEA-1 protein, as well as its colocalization with TfnR, which also utilizes the same entry pathway (66, 82). We compared the movement of integrin-binding type O_1Campos with that of the HS-binding variant O_1C3056R-KGE. To perform these experiments, we utilized COS-1 cells, since the anti-EEA-1 antibody did not react with this protein in MCF-10A cells. To be able to use COS-1 cells for this experiment, we demonstrated that the HS-binding virus utilized the same entry route into this cell line as it did into

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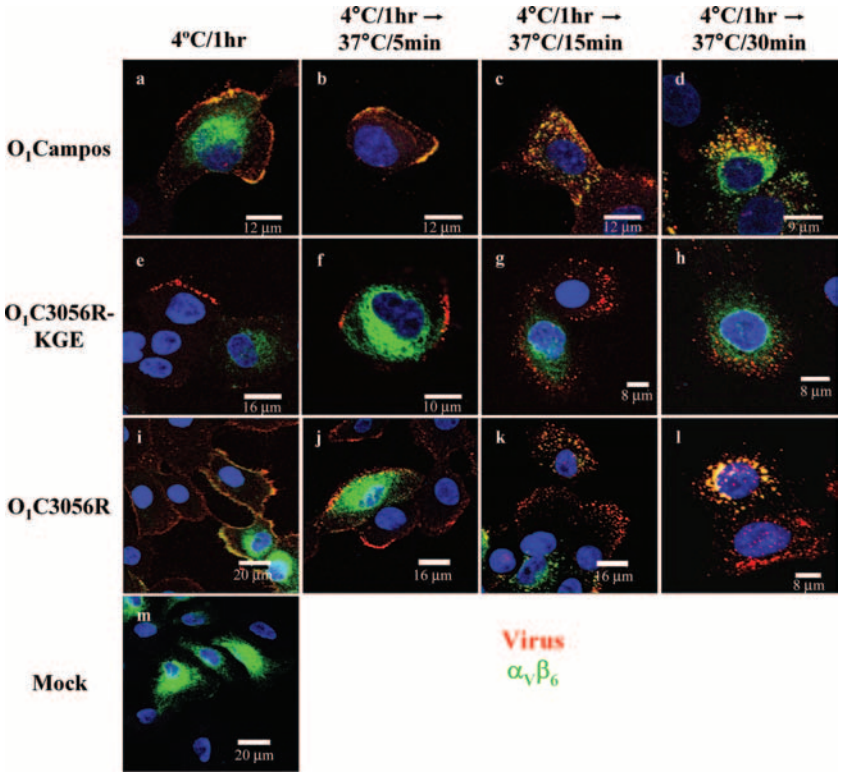

FIG. 2. Analysis of FMDV interaction with the $\alpha_V\beta_6$ integrin in MCF-10A cells. Cells were infected with type O_1Campos (a to d), type O_1C3056R-KGE (e to h), and type O_1C3056R (i to l) or were mock infected (m) at 4°C as described in Materials and Methods. Cells were fixed and probed for viral proteins (red) and the $\alpha_V\beta_6$ integrin (green), as described in Materials and Methods, immediately p.a. (a, e, and i) or at various times after cells were shifted to 37°C (b to d, f to h, and j to l). Only the merged photomicrographs are shown.

MCF-10A cells by looking at the colocalization of the virus with caveolae and cholera toxin (data not shown). Prior to infecting cells with type O_1Campos, we transfected these cells with plasmids encoding the α_V and β_6 integrin subunits as previously described (21). As we have shown previously (58), type O_1Campos colocalized with the early endosomal marker EEA-1 within 15 min after the temperature was shifted to 37°C (Fig. 4a). At 30 min postadsorption (p.a.), the virus did not colocalize with this protein, indicating that the virus had already been translocated from the early endosomes (Fig. 4b). In contrast, type O_1C3056R-KGE showed only partial colocalization with EEA-1 by 1 h after the temperature shift (Fig. 4c), and there were many cells in the culture that showed no colocalization of virus with EEA-1 (Fig. 4d). To try and resolve the temporal differences of viral movement through the endocytic system, we followed the internalization of both viruses along with that of TfnR, which traffics through early and recycling endosomes before returning to the cell surface (66, 82). Type O_1Campos rapidly began to colocalize with TfnR by 5 min p.a., and by 30 min p.a., there was almost complete colocalization of virus with the TfnR (Fig. 4e to g). In contrast, type O_1C3056R-KGE was only slightly colocalized with TfnR by 30 min p.a., but there was extensive colocalization by 2 h p.a. (Fig. 4h to j). These results suggested that integrin-binding and HS-binding viruses may traffic through different endocytic compartments. To analyze this further, we examined the association of the incoming viruses with two members of the Rab protein family, Rab4, a marker for early and recycling endosomes (72, 76, 83), and Rab11, a marker for recycling endosomes and the Golgi apparatus (75, 76, 87). The type O_1C3056R-KGE virus began to colocalized with Rab4 at between 15 min (not shown) and 30 min p.a. (Fig. 5a) and with Rab11 at 30 min p.a. (Fig. 5d), showing a complete colocalization with both markers by 1 h p.a. (Fig. 5b and e). In contrast, the integrin-binding type O_1Campos virus colocalized with Rab4 (not shown) but not with Rab11 (Fig. 5g to i).

In addition, we compared the entry of both viruses with that of fluorescent transferrin (Tfn-AF488), a well-established marker for the clathrin pathway, in the presence or absence of nocodazole, which blocks endosomal traffic from early to late endosomes by depolymerization of microtubules (32, 39). When the entry of the O_1C3056R-KGE virus and that of the integrin-binding virus were examined, colocalization with Tfn-AF488 by 30 min p.a. was observed for both, without showing any differences for the nocodazole-treated cells (not shown).

Type O_1C3056R-KGE also partially colocalized with the Golgi zone area protein (Fig. 6a and b) and COPI (Fig. 6c and d). We have previously shown that O_1Campos does not colocalize with proteins found in the Golgi apparatus (58). As we had previously shown for type O_1Campos (58), neither type O_1C3056R nor type O_1C3056R-KGE was delivered to the endoplasmic reticulum during internalization (not shown).

Taken together, these results indicate that integrin-binding viruses traffic only from the clathrin-coated vesicle to the early endosome, where virus uncoating presumably occurs. Viruses which utilize HS as a receptor traffic from the caveola through

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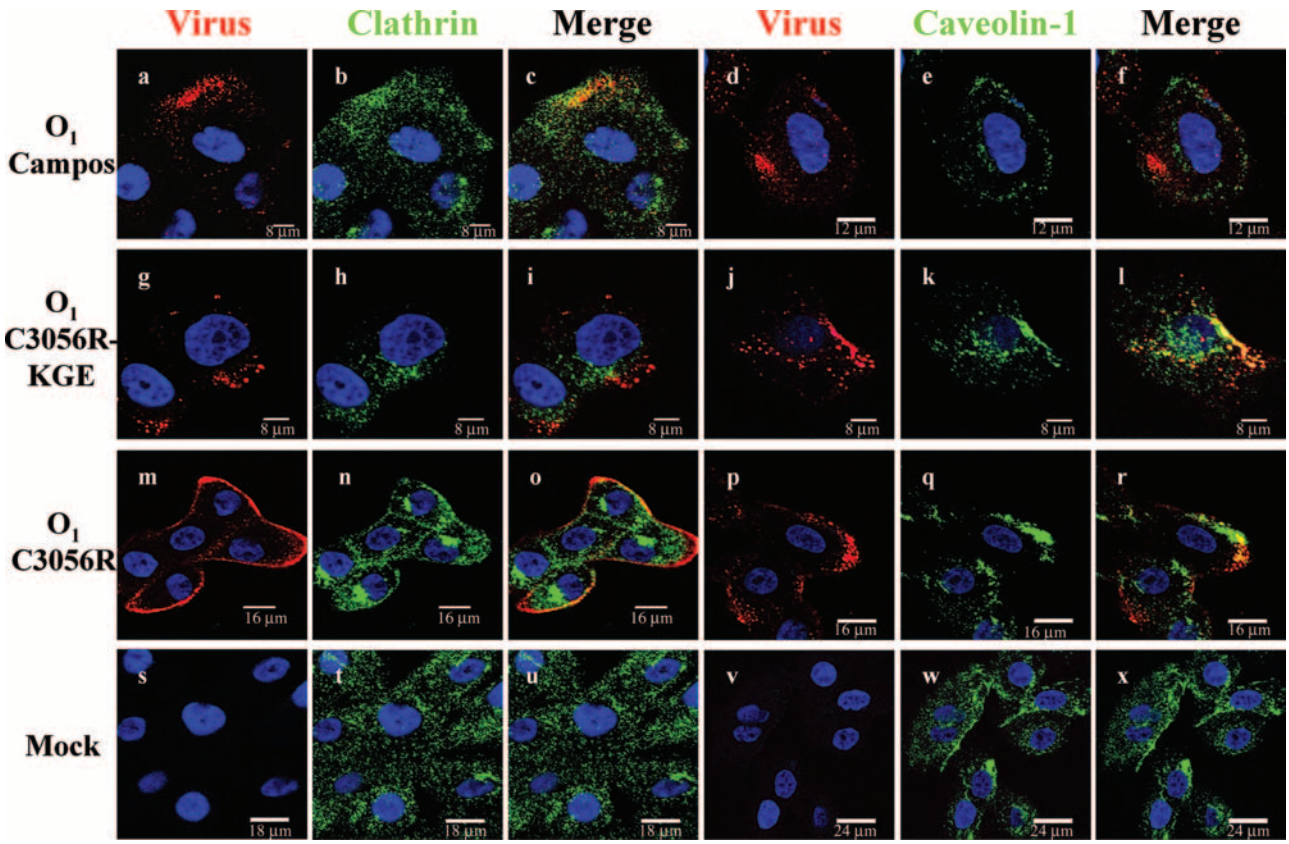

FIG. 3. Analysis of FMDV interaction with clathrin or caveolin-1 in MCF-10A cells. Cells were infected with type O_1Campos (a to f), O_1C3056R-KGE (g to l), and O_1C3056R (m to r) or were mock infected (s to x) at 4°C as described in Materials and Methods. Cells were fixed and probed, as described in Materials and Methods, for viral proteins (red), clathrin (green), or caveolin-1 (green) at 15 min after the temperature was shifted to 37°C.

the early endosome and into the recycling endosome and/or the Golgi apparatus.

Effects of inhibitors on the endocytic pathways on replication of integrin- and HS-binding FMDV. To confirm the microscopy analyses just presented, we analyzed viral replication in the presence of a number of inhibitors of endocytosis (Fig. 7). We had previously reported that chlorpromazine, an inhibitor of clathrin-mediated endocytosis (39, 85), inhibited type O_1Campos replication (58). In addition to inhibiting the integrin-binding virus, chlorpromazine also inhibited type O_1C3056R replication (not shown), but it did not inhibit the replication of type O_1C3056R-KGE, which utilizes only HS as the receptor and seems to be internalized via a caveola-mediated endocytosis pathway. Since the caveola-mediated entry of a number of picornaviruses is dependent on lipid rafts (20, 39, 42, 63, 79), we utilized three inhibitors of lipid raft formation, cytochalasin D (59), nystatin (1, 74), and nystatin plus progesterone. Not surprisingly, these inhibitors had no effect on the replication of O_1Campos (Fig. 7). MCF-10A cells treated for 2 h with nystatin or a combination of nystatin and progesterone resulted in an inhibition of O_1C3056R-KGE replication, which was also observed with confocal microscopy (not shown). To further analyze this result, we followed the entry of type O_1C3056R-KGE along with cholera toxin, which enters cells via caveolae and lipid raft-dependent endocytosis (33). Figure 8 show a colocalization of the HS-binding virus and cholera toxin AF488 starting at 15 min p.a. after the temperature was shifted to 37°C.

We had previously shown that late endosomes were not involved in the entry of type O_1Campos (58) and that the lack of effect of nocodazole, which causes the depolymerization of microtubules and inhibits translocation between peripheral early and late endosomes (10), on the replication of these viruses indicate that these viruses do not traffic through late endosomes. Amiloride, a compound which inhibits macropinocytosis, had no effect on viral replication of either of the viruses. In addition, brefeldin A, which disrupts the Golgi apparatus (30), had no effect on the replication of either the integrin- or the HS-binding virus (not shown), suggesting that the movement of type O_1C3056R-KGE to the Golgi apparatus (Fig. 6) might represent a dead-end pathway.

Finally, we examined viral replication in the presence of two compounds which raise the pH of endosomal vesicles, monensin and ammonium chloride. We previously showed that these compounds inhibited the replication of a number of different FMDV serotypes and subtypes by preventing the acid-induced breakdown of the viral capsid to pentamers, resulting in the release of the viral RNA (3). As observed for previous studies, monensin and ammonium chloride inhibited the replication of all three viral variants, O_1Campos, O_1C3056R-KGE (Fig. 7), and O_1C3056R (not shown).

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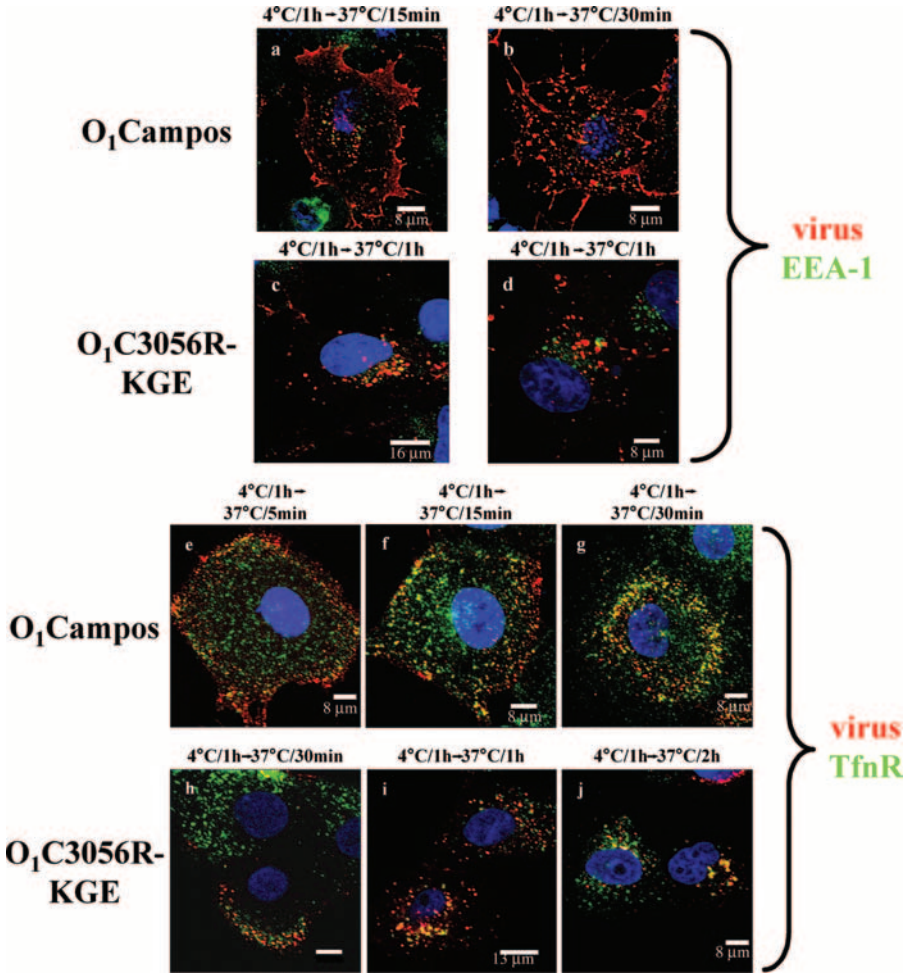

FIG. 4. Analysis of FMDV movement through early endosomes. COS-1 cells were infected with either type O_1Campos (a to b) or type O_1C3056R-KGE (c to d) at 4°C as described in Materials and Methods. MCF-10A cells were infected with either type O_1Campos (e to g) or type O_1C3056R-KGE (h to j) at 4°C as described in Materials and Methods. Cells were shifted to 37°C and, at the times indicated over each panel, were fixed and probed, as described in Materials and Methods, for viral proteins (red), EEA-1 (green), or TfnR (green). Only the merged photomicrographs are shown.

Inhibition of FMDV replication by caveolin-1 knockdown. To verify whether caveolin-1 plays a critical role in HS-binding virus entry, we tested the effect of a caveolin-1 knockdown on O_1Campos and O_1C3056R-KGE infection. Western blot analysis showed that the amount of caveolin-1 in MCF-10A cells could be reduced by 90% after cells were transfected with the caveolin-1 siRNA but did not change after they were transfected with a control siRNA (Fig. 9b). This reduction of caveolin-1 resulted in no inhibition of viral replication when the cells were incubated with O_1Campos (Fig. 9a). In contrast, when the cells were transfected with caveolin-1 siRNA and infected with the HS-binding virus O_1C3056R-KGE, an approximately 100-fold inhibition of replication was observed compared to that for the control (Fig. 9a). These results indicated that the expression of caveolin-1 is important and necessary for the infection by O_1C3056R-KGE.

DISCUSSION

FMDV is able to utilize a number of different cell surface molecules as viral receptors in cell culture, including at least four different α_V integrins and HS (8, 9, 36, 48). However, the viruses that use integrin-mediated binding are those responsible for disease in susceptible animals (52, 54, 70). It has recently been reported that these viruses enter cells via clathrin-mediated endocytosis (13, 58). The results we have presented in the present report indicate that FMDV O_1C3056R-KGE, which utilizes the alternative receptor HS, enters the cell via a caveola-mediated endocytic pathway. This conclusion is supported by the colocalization of HS-binding virus with caveolin-1 but not with the markers of the clathrin-mediated endocytosis, the inhibition of replication of the variant O_1C3056R-KGE but not the O_1Campos by caveolin-1 knockdown, and the sensitivity of HS-binding virus internalization to drugs that impair lipid raft components, such as nystatin and progesterone, or disturb anchorage of caveolae to the actin cytoskeleton, such as cytochalasin D. In addition, our results further suggest that the entry pathway of FMDV into cells is dependent on the receptor to which the virus binds.

To understand the HS-mediated entry of FMDV, we analyzed the internalization of the variant O_1C3056R-KGE, where

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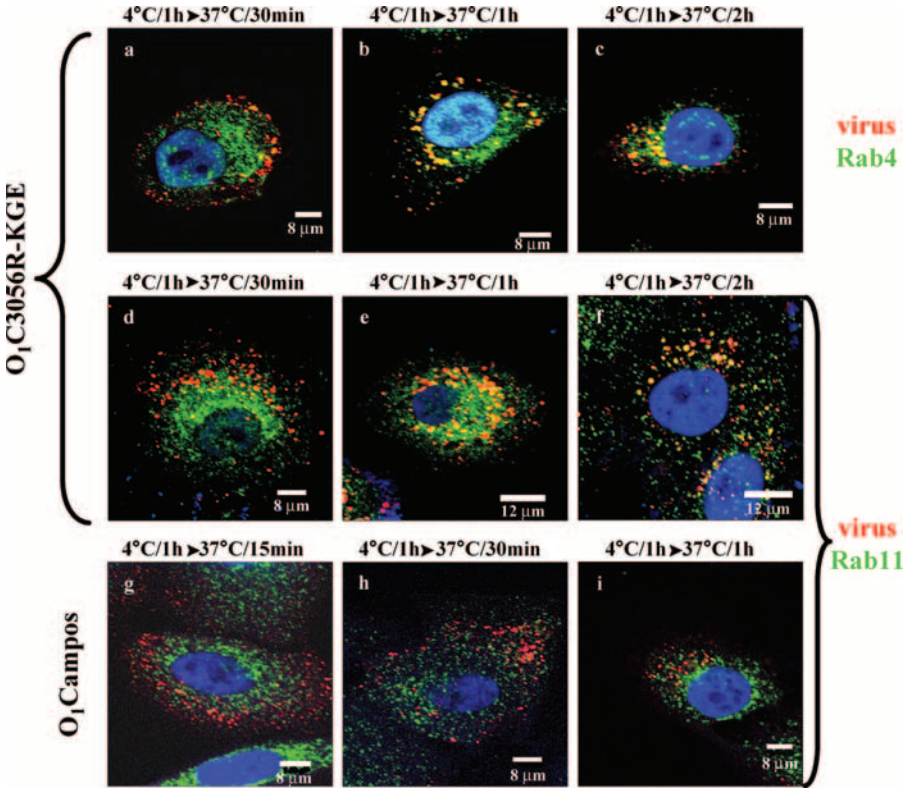

FIG. 5. Analysis of FMDV interactions with the Rab proteins in MCF-10A cells. Cells were infected with either type $O_1$3056R-KGE (a to f) or type O_1Campos (g to i) at 4°C as described in Materials and Methods. Cells were shifted to 37°C and, at the times indicated over each panel, were fixed and probed, as described in Materials and Methods, for viral proteins (red), Rab4 (green, a to c), or Rab11 (green, d to i). Only the merged photomicrographs are shown.

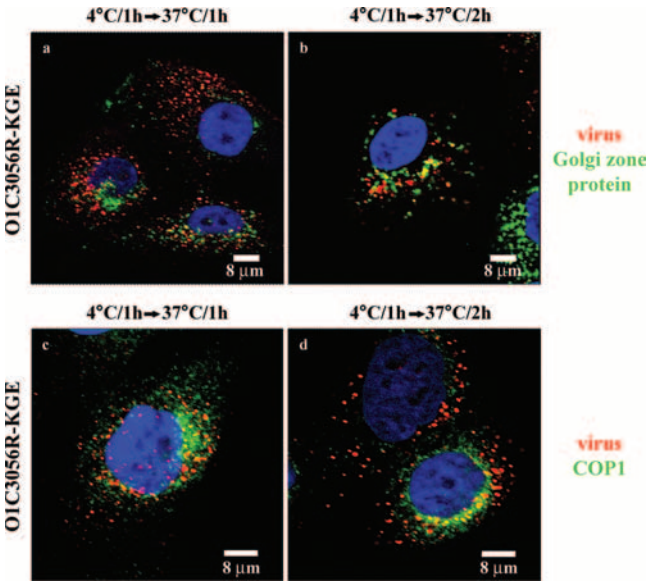

FIG. 6. Analysis of HS-binding FMDV with the Golgi apparatus in MCF-10A cells. Cells were infected with type $O_1$3056R-KGE at 4°C as described in Materials and Methods. Cells were shifted to 37°C and, at the times indicated over each panel, were fixed and probed, as described in Materials and Methods, for viral proteins (red), Golgi zone protein (green, a to b), or COPI (green, c to d). Only the merged photomicrographs are shown.

the RGD sequence of the G-H loop responsible for targeting the virus to integrins has been mutated to KGE and can utilize only HS as a receptor, and a second variant of type O_1Campos, O_1C3056R, which can bind to both HS and integrin receptors (8, 54). For most of our studies, we utilized the human breast epithelial cell line MCF-10A, which is a heterogeneous population of cells, some of which express the $\alpha_V\beta_3$ or $\alpha_V\beta_6$ integrin receptors (58).

All three of the viruses replicated in the MCF-10A cells (Fig. 1), and the analysis of viral binding shows that O_1Campos bound only to cells expressing the $\alpha_V\beta_6$ integrin (Fig. 2), as reported previously (58). In contrast, type O_1C3056R was bound to cells which either did or did not express the integrin and colocalized with the integrin in expressing cells (Fig. 2). Type O_1C3056R-KGE bound to cells irrespective of their integrin expression and never colocalized with the integrin (Fig. 2). Treatment of cells with heparinase abolished binding of the O_1C3056R-KGE virus (not shown). Thus, this virus bound only to the HS receptor, while the O_1C3056R variant bound to either HS or the integrin. We followed the entry of these viruses along with markers for clathrin and caveola-mediated entry. As has been shown previously (13, 58), integrin-binding virus colocalized with clathrin upon entering the cell (Fig. 3), while the HS-binding virus appeared to colocalize with caveolin-1 (Fig. 3), indicating that the HS-virus complex was entering via the caveolae. The viral variant which bound to both integrin and HS appeared to colocalize with both clathrin and

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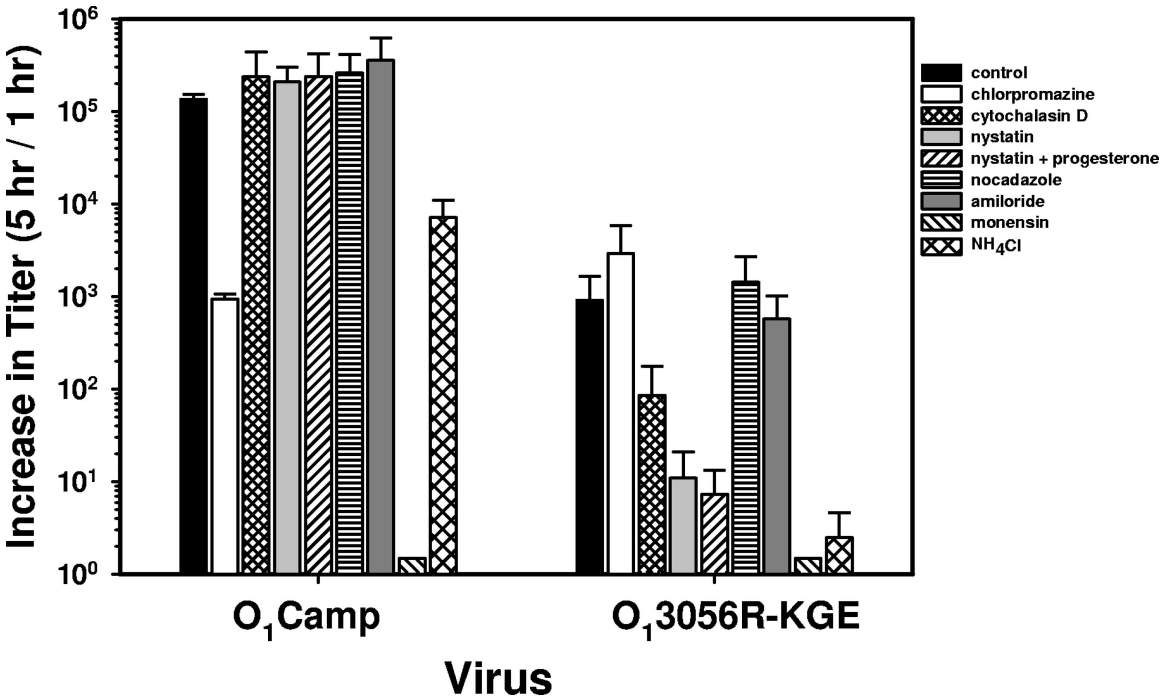

FIG. 7. Effect of inhibitors of internalization on FMDV replication in MCF-10A cells. Cells were treated with each of the inhibitors and infected with the O_1Campos or $O_1$3056R-KGE variant, as described in Materials and Methods. Immediately p.a., one set of plates was frozen at −70°C and a second set of plates was frozen at −70°C at 5 h after infection. Plaque titers were determined in BHK-21 cells and are represented as the ratio of plaque titers at 1 to those at 5 h p.i.

caveolin-1 (Fig. 3). We confirmed these results by using the inhibitor of clathrin-mediated entry, chlorpromazine. This compound inhibited the replication of both type O_1Campos (Fig. 7) and type O_1C3056R (not shown); however, the latter virus was inhibited to a lesser extent. In contrast, chlorpromazine had no effect on the replication of type O_1C3056R-KGE (Fig. 7).

Caveolae are a type of lipid raft present on the cell surface which contain the caveolin protein (69). Caveolin is a cholesterol-binding protein (53, 80), and caveolae are membrane domains that are cholesterol dependent (80). The replication of two other nonenveloped viruses, simian virus 40 (SV40) and echovirus type 1 (EV1), which enter cells via caveolae (62, 63), are inhibited by compounds that deplete cholesterol and disrupt lipid rafts (1, 63). In the presence of nystatin and progesterone, compounds that are cholesterol-sequestering and lipid-raft disrupting (1, 40, 74), O_1C3056R-KGE viral infection was inhibited (Fig. 7). We also found that the replication of the HS-binding virus was inhibit to a lesser extent by cytochalasin D, which disrupts the actin cytoskeleton and inhibits lipid raft-mediated internalization (18, 59). For EV1, it has been demonstrated that cytochalasin D does not inhibit viral replication (63), suggesting that EV1, may enter in an actin-independent manner. The authors of that study, however, speculated that this result may depend on the cell type used (63). Disruption of the microtubules with the drug nocodazole had no effect on the

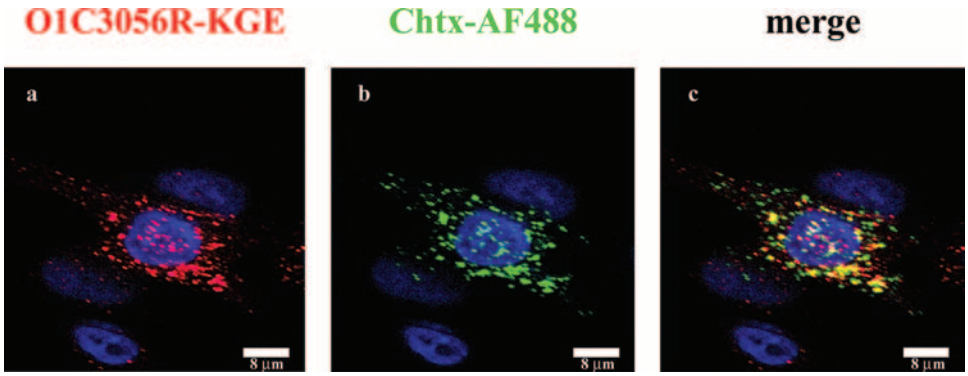

FIG. 8. Internalization of type $O_1$3056R-KGE and fluorescent cholera toxin in MCF-10A cells. Cells were infected with virus in the presence of Alexa Fluor 488-labeled cholera toxin as described in Materials and Methods. Cells were fixed 15 min after infection, and after they were shifted to 37°C, they were probed for virus (red). Cholera toxin was detected as Alex Fluor 488 (green).

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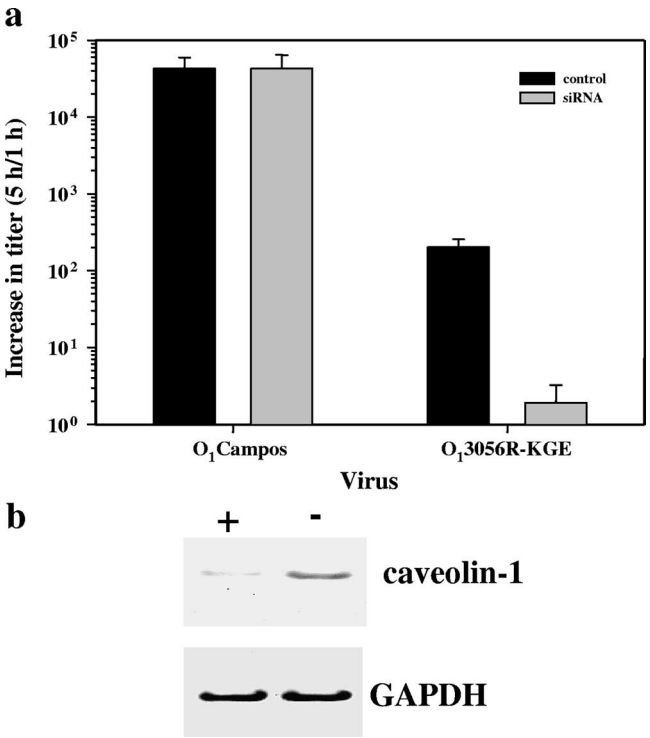

FIG. 9. Viral yields from FMDV infections in MCF-10A cells treated with siRNA to reduce the intracellular concentration of caveolin-1. (a) MCF-10A cells transfected with four RNA duplexes targeted to caveolin-1 or with a control siRNA, siGlo, for 48 h at 37°C, as described in Materials and Methods. Triplicate plates were infected with FMDV at an MOI of 1 PFU/cell for the indicated times. Plaque titers were determined in BHK-21 cells and are represented as the ratios of plaque titers at 1 to those at 5 h p.i. (b) The relative abundance of caveolin-1 protein in the cells treated with the control or that with cells with the caveolin-1-targeted siRNA duplexes was determined by immunoblotting using polyclonal antibodies to caveolin-1 and GAPDH.

replication of either of the viruses, O_1Campos or O_1C3056R-KGE, which indicates that these viruses do not traffic from early to late endosomes.

To verify that caveolae play a critical role, we tested the effect of a caveolin-1 knockdown on O_1Campos and O_1C3056R-KGE infection. When the abundance of the caveolin-1 protein was reduced by more than 90% by siRNA, an inhibitory effect on viral replication was observed for the O_1C3056R-KGE HS-binding virus, indicating that the expression of caveolin-1 is important and necessary for infection by this virus, while the caveolin-1 knockdown had no effect on the replication of O_1Campos.

To further define the role of lipid rafts in the internalization of type O_1C3056R-KGE, we analyzed virus entry along with that of cholera toxin. Cholera toxin has been shown to be associated with lipid rafts (56, 57, 73) and enters cells partially through caveolae (55); therefore, cholera toxin can be used as a marker for lipid rafts. By using fluorescence-labeled cholera toxin, we were able to follow the internalization of the virus. We observed that the O_1C3056R-KGE virus colocalizes with cholera toxin on entering the cell (Fig. 8), indicating again that HS-binding virus enters the cell in a structure containing caveolin-1 which is dependent on cholesterol. It has been shown that cholera toxin binds to GM1 ganglioside and partially passes through caveosomes during transport to the Golgi complex (55), suggesting that the HS-binding virus may be entering caveosomes at late stages of infection.

It has been demonstrated that the dynamics of the caveolar endocytosis pathway are slow compared with those of the clathrin-endocytosis pathway (24, 81, 84). This is in agreement with our results, since we found that the HS-binding FMDV moves through the endocytic pathway at a slower rate than that of the integrin-binding virus that uses the clathrin-endocytosis pathway.

We had previously suggested that type O_1Campos enters the cell in an early endosome, followed by movement into a recycling endosome, where the virus breaks down due to the acidic environment, releasing the RNA (58). The results presented in this work indicate that the integrin-binding virus enters into the early endosomes, where breakdown presumably occurs. These results are based on following the entry of virus in association with Rab4 and Rab11. The Rab proteins are small GTPases, members of the Ras superfamily, which are distributed and regulate transport through intracellular organelles (see 75, 78, and 88 and references therein). Rab4 regulates endocytic recycling to the plasma membrane and is found on both early and recycling endosomes (72, 76, 83), while Rab11 is found on recycling endosomes and the Golgi apparatus and regulates recycling through the recycling endosomes and Golgi-plasma membrane traffic (75, 76, 87). We found that both variants, O_1Campos and O_1C3056R-KGE, colocalized with the early endosomal marker EEA-1, TfnR, and Rab4 (Fig. 4 and 5), indicating that both variants trafficked into the early endosomes. Interestingly, only the HS-binding virus colocalized with Rab11 (Fig. 5) and colocalized partially with the Golgi zone area protein and the COPI protein (Fig. 6), which is part of a complex of proteins involved in intra-Golgi apparatus transport (64). Thus, we believe that the HS-binding viruses enter the cell through caveolae and traffic through early endosomes and into recycling endosomes, where the breakdown and release of the RNA may occur. Our results are consistent with several other studies demonstrating that cargo internalized by caveolae and markers of the clathrin-endocytosis pathway merge at early endosomes, with the detection of caveolin-1 on early endosomes. These results suggest that cargo and membranes internalized through caveolae are predominantly transported to the early endosomes for further sorting and delivery to cellular locations, although exceptions may occur, depending on the cell type (60, 61, 71).

We cannot rule out the possibility that this virus also traffics through the Golgi apparatus; however, brefeldin A, which disrupts the Golgi apparatus (30), did not inhibit the replication of either the integrin-binding or the HS-binding virus (not shown). Moreover, a colocalization of the HS-binding virus with cholera toxin, which is known to traffic to the Golgi complex, was observed at late stages of infection. Taken together, these results suggest that traffic through the Golgi apparatus, if it occurs, may be a dead-end pathway.

We, and others have previously reported that monensin, a compound which raises the pH of endocytic vesicles, inhibited the replication of a wide variety of FMDV serotypes and subtypes by preventing the acid-induced breakdown of virus particles, result-

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ing in genome release (3, 15). We also recently reported that in the presence of monensin, integrin-binding viruses were not released from the early endosomal compartment (58). In this study, pretreatment of cells with monensin inhibited the replication of both viruses, O_1Campos and the variant O_1C3056R-KGE. Monensin probably raises the pH of both the early and the recycling endosomes enough to prevent viral uncoating and therefore inhibits the replication of the viruses. Similar results were observed when cells were pretreated with ammonium chloride, although there was a greater effect on the replication of the O_1C3056R-KGE variant (Fig. 7).

Our studies were undertaken to identify targets for antiviral intervention to control FMD. Even though it has been shown that the HS-binding viruses are relatively avirulent (70), it is important to determine if FMDV can enter cells via alternative mechanisms. FMDV has been shown to be remarkably resilient and variable in the field, and the emergence of new viral variants, which might manifest different modes of viral entry, needs to be addressed.

ACKNOWLEDGMENTS

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