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Productive entry of type C foot-and-mouth disease virus into susceptible cultured cells requires clathrin and is dependent on the presence of plasma membrane cholesterol

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Abstract

We have characterized the entry leading to productive infection of a type C FMDV in two cell lines widely used for virus growth, BHK-21 and IBRS-2. Inhibition of clathrin-mediated endocytosis by sucrose treatment decreased both cell entry and virus multiplication. Evidence of a direct requirement of clathrin for productive viral entry was obtained using BHK21-tTA/anti-CHC cells, which showed a significant reduction of viral entry and infection when the synthesis and functionality of clathrin heavy chain was inhibited (Tet− cells). This was also observed for vesicular stomatitis virus (VSV) productive entry. The effect of NH_4Cl and concanamycin A on FMDV entry and infection was consistent with the requirement of acidic compartments for decapsidation and virus replication. As expected from its higher stability at acidic pH, this requirement was higher for VSV. Since BHK-21 and IBRS-2 cells expressed caveolin-1, we explored the effect on productive virus entry of drugs that interfere with caveolae-mediated endocytosis. Treatment with nystatin did not reduce entry and infection of FMDV or VSV, while cholesterol depletion with MβCD significantly inhibited both steps of the FMDV cycle, indicating that plasma membrane cholesterol is required for virus productive entry.

Introduction

The way in which different viruses can exploit endocytic pathways to enter susceptible cells is varied and provides of a useful tool to gain basic information on the molecular mechanisms involved in membrane traffic in animal cells (Dimitrov, 2004; Marsh and Helenius, 2006). Although the clathrin-dependent pathway is the entry route best characterized for virus entry, alternative, clathrin-independent pathways are also used. These alternative pathways can be classified into two major classes: lipid-raft-dependent pathways (including the caveolar pathway) used by acid-independent viruses, and non-clathrin, non-caveolae-mediated endocytosis (Marsh and Helenius, 2006; Sieczkarski and Whittaker, 2005).

Most viruses do need endocytic internalization for penetration and productive infection. A dependence on low pH for penetration drives viruses to use the decreasing pH of endocytic organelles as a cue to activate the penetration reactions and allows viral escape to the cytoplasm at specific locations before the virus is delivered to the hydrolytic lysosomes. In addition, no viral components remain on the cell surface after penetration for detection by host immune defences (Smith and Helenius, 2004). However, viruses that do not require low pH for penetration can be found in different families of non-enveloped viruses, including Picornaviruses, such as some enteroviruses (Pietiainen et al., 2004). Moreover, it has been proposed that some enteroviruses, such as poliovirus (PV), may penetrate directly through the plasma membrane, though this remains contentious (Bubeck et al., 2005; Hogle, 2002).

The Picornaviridae family comprises a wide number of important human and animal pathogens. In spite of their close structural relationships, different members of this family can

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use a variety of entry routes into host cells (DeTulleo and Kirchhausen, 1998; Joki-Korpela et al., 2001; Marjomaki et al., 2002; Snyers et al., 2003; Stuart et al., 2002). Picornaviruses are known to undergo structural alterations during receptor interactions and entry, leading to the uncoating of the virus particle and release of the viral genome into the cytoplasm. This is required for subsequent translation and replication events (Hogle, 2002). pH stability of different Picornaviruses could be very important for uncoating. The acidic pH environment of endosomes or their rupture has been proposed as the mechanisms utilized by pH-sensitive virus (Huber et al., 2001; Schober et al., 1998), while uncoating of acid pH-stable virus takes place in other organelles (Pietiainen et al., 2004).

Foot-and-mouth disease virus (FMDV) is the prototype member of the aphthovirus genus within the family Picornaviridae (Rowlands, 2003; Sobrino and D.E., 2004). The virus causes a vesicular disease of cloven-hoofed animals that produces high losses in livestock production and trading (Sutmoller et al., 2003). FMDV exhibits a high genetic and antigenic variability, which is reflected in seven serotypes and multiple variants, that endows this virus with a high potential for adaptation (Domingo et al., 2003). FMDV particles are composed of a non-enveloped capsid containing a linear plus strand RNA genome about 8.500 nucleotides long. This RNA encodes a single polyprotein, which is expressed and cleavaged in infected cells to render capsid proteins (VP1 to 4) as well as 15 different mature non-structural proteins (Belsham, 2005). FMDV can initiate infection of cultured cells via different α_v integrins (Berinstein et al., 1995; Jackson et al., 2004; Jackson et al., 2002; Jackson et al., 2000). Viruses that are infectious in vivo can enter cultured cells using $\alpha_v\beta_3$ and $\alpha_v\beta_6$ integrins (Jackson et al., 2000; Neff et al., 1998). This latter integrin is expressed constitutively on the epithelial cells targeted by FMDV in cattle, and it has been suggested to be the major in vivo receptor for this virus (Monaghan et al., 2005). Interaction of FMDV with these integrins requires an Arg-Gly-Asp (RGD) triplet located at the G-H loop of capsid protein VP1 (Acharya et al., 1989), which is also a main antigenic site involved in the interaction with neutralizing antibodies (Hewat et al., 1997; Mateu, 1995; Verdaguer et al., 1995).

Recent reports have approached the analysis of the early internalization events mainly by using human cell lines transfected with or constitutively expressing human integrins $\alpha_v\beta_3$ or $\alpha_v\beta_6$ (Berryman et al., 2005; O'Donnell et al., 2005). In both cases, the use of specific inhibitors and co-localization assays with cellular markers indicated that FMDV types A and O utilize a clathrin-dependent mechanism to infect these cells. This was further supported by the inhibition of infected cells observed upon expression of a dominant-negative version of AP180 that is required for clathrin cage assembly (Berryman et al., 2005). The infection reduction observed upon treatment with inhibitors of the early to late endosomal traffic, indicated also that infection occurred from early endosomes (Berryman et al., 2005), which is consistent with the high instability of FMDV capsids at pH below 7 (Curry et al., 1995; van Vlijmen et al., 1998).

In this study, two cell lines (BHK-21 and IBRS-2), widely used for basic FMDV studies, as well as for vaccine production and diagnostic purposes (Barteling, 2004; Brown, 1998), have been used to characterize the requirements for internalization leading to productive infection of a type C FMDV isolate and of VSV, a rhabdovirus causing a vesicular animal disease clinically similar to FMD, whose entry through clathrin-mediated endocytosis is well characterized (Matlin et al., 1982; Sun et al., 2005). Evidences are presented on the involvement of clathrin in productive type C FMDV endocytosis by BHK-21 and IBRS-2 cells, and of the direct requirement of clathrin heavy chain (CHC) for this process in BHK21-tTA/anti-CHC cells expressing low levels of a non-functional CHC (Iversen et al., 2003). In addition, treatment of BHK-21 and IBRS-2 cells with the cholesterol-depleting drug MβCD has revealed a dependence on plasma membrane cholesterol for FMDV entry and growth.

Results

Hypertonic shock inhibits type C FMDV infection in BHK-21 and IBRS-2 cells

Treatment with sucrose has been shown to block uptake and infection by virus that use clathrin-dependent endocytosis to entry into cells (Gilbert and Benjamin, 2000; Nicola et al., 2003) and to inhibit the uptake of type O FMDV isolates in SW480-$\alpha_v\beta_6$ cells (Berryman et al., 2005). BHK-21 and IBRS-2 cells (Fig. S1) treated with 0.45 M sucrose showed a drastic inhibition of TF internalization, a ligand whose uptake takes place by clathrin-mediated endocytosis (Sandvig et al., 1987), as well a reduced entry of type C FMDV in BHK-21 cells, whose fluorescence appeared mostly distributed at the plasma membrane (Fig. 1A). This inhibition of FMDV endocytosis correlated with a decrease in the progress of virus infection in experiments performed at low MOI, to limit the possibility of unspecific viral uptakes due to the high amount of virus used in the internalization assays. Thus, a 65% of plaque reduction was observed in BHK-21 cells treated with sucrose, relative to control cells (Fig. 1B). Likewise, detection of infected cells by immunofluorescence with a MAb to capsid protein VP1 showed that sucrose treatment significantly reduced the percentage of BHK-21 and IBRS-2-infected cells by 91% and 81%, respectively (Fig. 1C). The hypertonic shock inhibited in a similar amount the total virus yield recovered from BHK-21 or IBRS-2-infected cultures (Fig. 1C). These decreases in virus-infected cells and virus production correlated with a reduction of the amount of capsid protein VP1 detected by western blot of BHK-21-infected cell extracts (Fig. 1D).

Thus, these results are consistent with the requirement of clathrin-dependent endocytosis for type C FMDV internalization leading to virus growth.

Expression of clathrin is required for FMDV infection

Sucrose treatment might inhibit endocytic pathways other than the clathrin-dependent (Roseberry and Hosey, 2001).

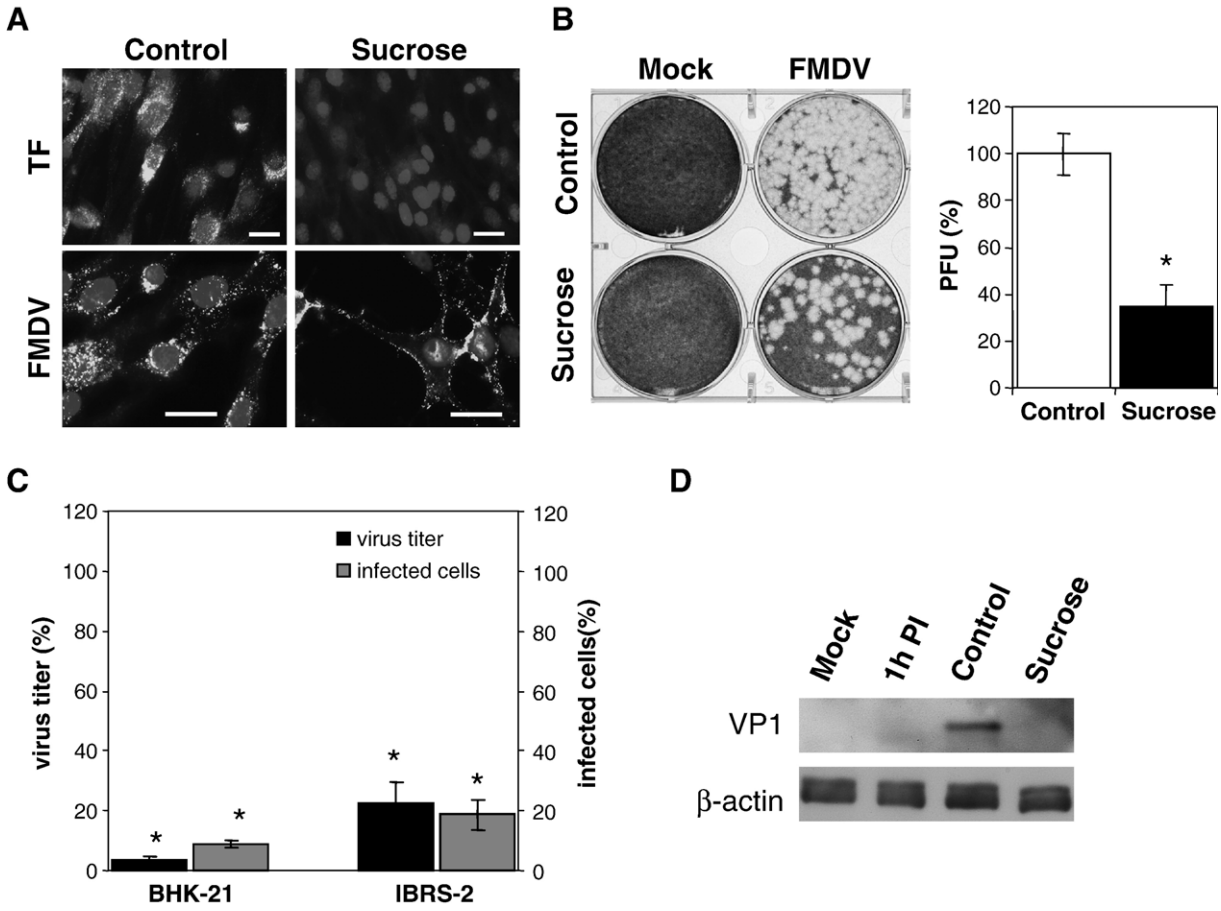


Fig. 1. Hypertonic medium inhibits FMDV internalization and infection of BHK-21 and IBRS-2 cells. (A) Sucrose treatment inhibits TF endocytosis and FMDV internalization into BHK-21 cells. Cells, incubated in hypertonic medium for 30 min, were either infected with FMDV (MOI of 35) for 25 min or incubated with Alexa Fluor 488-labeled TF. Extracellular TF was eliminated by acid wash and FMDV was detected using MAb 5C4 and a secondary antibody coupled to Alexa Fluor 488. Scale bars 20 μm. (B) FMDV plaque reduction assay in BHK-21 cells. Cells treated, or not, with sucrose were infected with FMDV (50–100 PFU) and the PFU determined as described in Materials and methods (left). The percentage of PFU recovered is relative to those of observed in untreated control cells. The means and SD from three independent experiments are shown (right). (C) Sucrose treatment inhibits FMDV infection. Sucrose treated cells were infected with FMDV (MOI of 0.5). The total virus titer at 8 h PI was determined by plaque assay on BHK-21 cells. Infected cells (6 h PI) were estimated as those positive for immunofluorescence using MAb SD6 and an Alexa Fluor 488 as secondary antibody. The percentages of virus titers and of immunofluorescent cells are relative to those observed in untreated control cells. The means and SD from three independent experiments are shown. (D) Hypertonic medium reduces FMDV VP1 expression. BHK-21 cells treated as in panel C were lysed at 6 h PI and VP1 was detected by western blot assay using MAb SD6. Blotting to an anti-β-actin MAb was included as control of protein loading.

Therefore, the direct involvement of clathrin in FMDV entry and virus multiplication was further analyzed by using a BHK-21 derived cell line (BHK21-tTA/antiCHC) stably transfected with an antisense RNA to the CHC. Transcription of this antisense RNA from a tetracycline-repressible promoter, not only inhibits expression of new CHC but induces a lack of functionality in the previously synthesized CHC molecules (Iversen et al., 2003). Type C FMDV-infected BHK21-tTA/antiCHC cells grown in the presence of tetracycline (Tet+). FMDV infection of these cells did not induce a clear cytopathic effect (data not shown). However, an increase of virus detection in the infection medium was found as infection progressed (Fig. 2A). The viral titres recovered, which ranged from 10^3 to 10^4 PFU/ml, were lower than those found in BHK-21-infected cells. Infection of Tet+ cells with VSV produced cytopathic effect and a parallel kinetic in virus production was found in the culture medium (Fig. 2A). This rhabdovirus was chosen as control in these experiments since its clathrin requirement for cell entry is well established (Sun et al., 2005). In this case, viral titres were higher than those recovered upon FMDV infection (Fig. 2A).

Removal, of tetracycline from BHK21-tTA/antiCHC culture medium (Tet−) resulted in about a 70% reduction, detected by western blot of cell extracts (Fig. 2B), of a non-functional CHC (Iversen et al., 2003). As expected, a decrease in clathrin-mediated endocytosis (Fig. 2C) was observed in these cells. Thus, Tet− cells positive for TF fluorescence represented only a 10% of those found in Tet+ cells (Fig. 2D). This lack of functional clathrin inhibited both FMDV and VSV internalization, as shown in Fig. 3A. Tet− cells mostly accumulated viral fluorescence in the plasma membrane, which appeared with a perinuclear distribution in FMDV-infected Tet+ cells. In addition, a reduction of 67% in the PFU recovered from Tet− cells infected with VSV was observed and the size of the viral plaques developed was considerably smaller (Fig. 3B). Likewise, the percentage of Tet− infected cells positive to G glycoprotein fluorescence was lower (51%) than that detected in

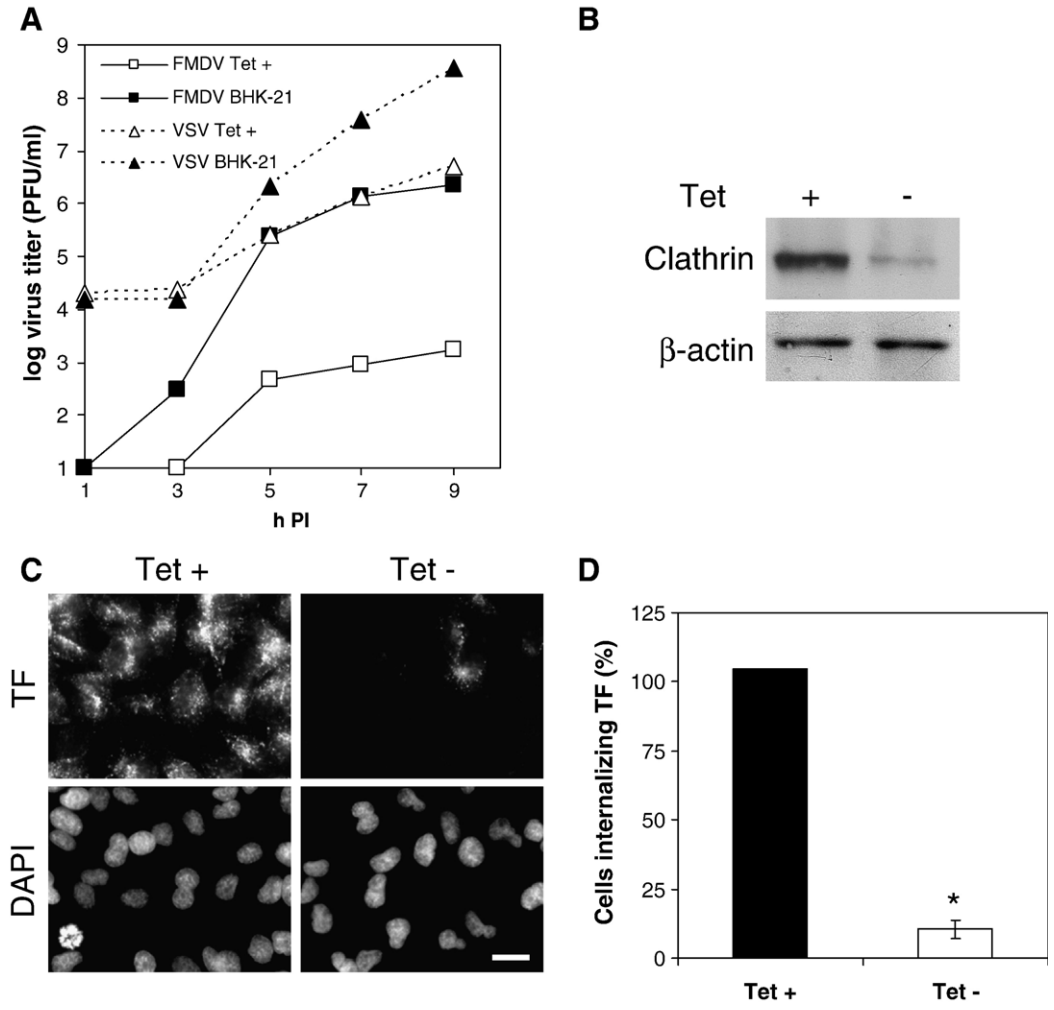


Fig. 2. Susceptibility of Tet+ cells to FMDV and VSV infection and effect of clathrin depletion in TF uptake. (A) Growth curve of FMDV and VSV in BHK-21 and Tet+ cells, which were infected at an MOI of 0.5. Virus titre in infection medium was determined, at different time points, by plaque assay on BHK-21 cells. The means of two independent experiments are shown; SD values were lower than 25%. (B) Western blot with an antiserum to CHC in Tet+ and Tet− cells. Blotting to an anti-β-actin MAb was included as control of protein loading. (C) Expression of CHC antisense RNA inhibits TF internalization. Tet+ and Tet− cells were incubated with Alexa Fluor 488-labeled TF, as described in Materials and methods. Scale bar: 20 μm. (D) Expression of CHC antisense RNA reduces the number of cells internalizing TF. The percentage of cells (relative to values of Tet+ cells) positive for TF fluorescence, determined as in panel C, is indicated. The means and SD from three independent experiments are shown.

Tet+ cells (Fig. 3C). However, infection of Tet− cells with FMDV did not result in detectable viral plaques and the low level of VP1 fluorescence impaired a sensitive detection of infected cells (data not shown).

To analyze virus production in Tet+ and Tet− infected cells, total virus production was titrated in BHK-21 cells. A significant reduction in FMDV titres (about 80%) was found in Tet− cells, which was similar to that observed in this cells infected with VSV (Fig. 3D).

These results confirm the direct requirement of clathrin for productive type C FMDV internalization in these BHK-21 derived cells.

Progress of FMDV infection depends on endosomal acidification

To determine the requirement of trafficking through acidic endosomes for FMDV infection progress, cells were incubated with NH_4Cl, which blocks endosomal acidification and has been shown to inhibit FMDV and VSV growth (Baxt, 1987; Carrillo et al., 1984; Matlin et al., 1982). BHK-21 cells stained with AO showed a vesicular pattern of red fluorescence, corresponding to accumulation of AO in acidic endosomes. In contrast, and confirming the increase in endosomal pH induced by NH_4Cl, when cell cultures were incubated with 25 mM NH_4Cl, a drastic reduction of red fluorescence was observed, which was complete in cells treated with 50 mM NH_4Cl (Fig. 4A, a–c). The effect of this inhibition of endosomal acidification on FMDV infection is shown in Fig. 4A, d–f. Control cells infected with FMDV at a high MOI showed, at 3 h PI, a VP1 fluorescence pattern distributed throughout cell cytoplasm, corresponding to the novo VP1 synthesis, as expected from the progress of virus infection. In contrast, infected cells treated with NH_4Cl exhibited a VP1 fluorescence that appeared with a vesicular pattern, which resembled that observed in cells at early

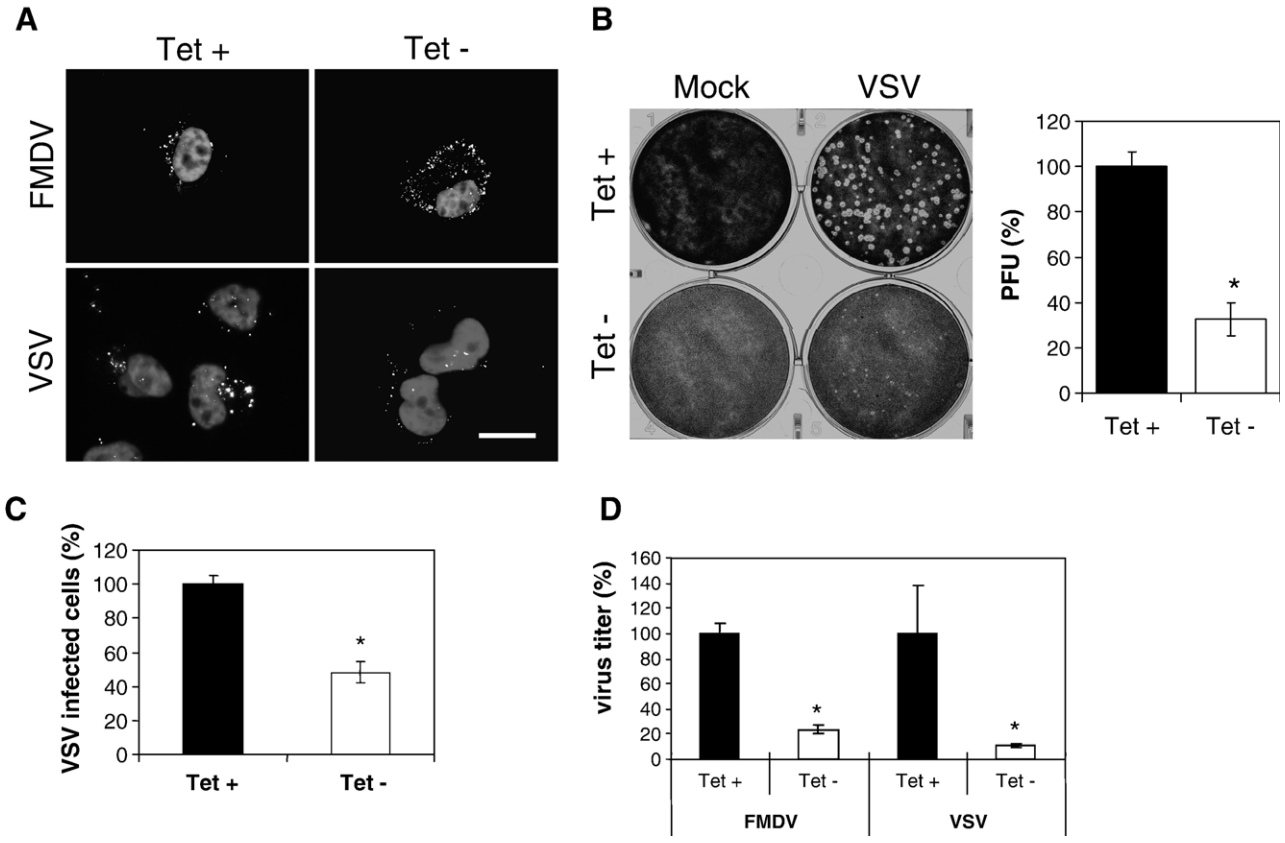


Fig. 3. Expression of CHC antisense RNA inhibits FMDV and VSV internalization and infection in Tet− cells. (A) Tet+ and Tet− cells were infected with FMDV or VSV (MOI of 35); after 25 min, cells were fixed and processed for immunofluorescence using MAb 5C4 or MAb I1, respectively. Scale bar: 20 μm. (B) PFU recovered from Tet+ or Tet− cells infected with 50–100 PFU of VSV. The means and SD from three independent experiments are shown (right). (C) Expression of CHC antisense RNA reduces the number of VSV-infected cells. Tet+ or Tet− cells were infected with VSV (MOI of 0.5). At 6 h PI, cells were fixed and processed for immunofluorescence using MAb I1. The means and SD from three independent experiments are shown. (D) Expression of CHC antisense RNA reduces FMDV and VSV virus yield. Tet+ and Tet− cells were infected with FMDV or VSV (MOI of 0.5) and the virus yield at 9 h PI was determined by plaque assay in BHK-21 cells. The means and SD from three independent experiments are shown. The percentages shown in panels B–D are relative to the values of Tet+ cells.

times upon infection (see Fig. 1A). A parallel effect of NH_4Cl treatment was found in FMDV-infected IBRS-2 cells (see Fig. S2). As reported (Rigaut et al., 1991), treatment of BHK-21 cells with NH_4Cl blocked VSV entry progress in a comparable manner (Fig. 4A, g–i).

The blockage observed in virus progression correlated with a significant decrease in the percentage of cells expressing FMDV capsid proteins, which was reduced to values below 20% of those observed in control BHK-21 cells, being this reduction higher in IBRS-2 cells (Fig. 4B). A significant reduction in the VP1 protein detected by western blotting of BHK-21 and IBRS-2-infected cell proteins was also noticed (not shown). Likewise, the total virus yield from BHK-21 treated cells was, at least, ten-fold lower than those recovered from control cells (Fig. 4B), and again higher reductions were found in IBRS-2 cells. The susceptibility of VSV infection to NH_4Cl was higher and resulted in about a 4 log reduction of the virus yield. Treatment with NH_4Cl did not inhibit the virus yield when added to the cultures 3 h PI (data not shown), ruling out the possibility that NH_4Cl significantly affects viral replication, as reported (Baxt, 1987).

As described for type O FMDV (Berryman et al., 2005), raise of endosomal pH by pre-treatment with concanamycin A, a specific inhibitor of V-ATPAse, inhibited FMDV and, to a larger extent, VSV infection (see Fig. S3). These inhibitions were lower than those exerted by NH_4Cl, which could be due to pH recovery after concanamycin A removal (see Materials and methods). As observed with NH_4Cl, concanamycin A also induced a higher reduction in virus titers in FMDV-infected IBRS-2 cells for which inhibitions of about 90% were observed with 100 nM concentrations of the drug.

Thus, these results support that traffic through acidic compartments is required for type C FMDV decapsidation and virus replication. As expected from its higher stability to acidic pH, this requirement was higher for VSV replication.

Requirement of acidic endosomes for FMDV infection in Tet− cells expressing non-functional clathrin

Treatment of Tet+ cells with NH_4Cl resulted in 70% reduction of the virus recovered upon FMDV infection relative to control Tet+ cells (Fig. 5A). Conversely, no differences in the virus yield was found between Tet− cells, either treated or not with NH_4Cl, whose viral titers were similar to those found in Tet+ cells treated with this compound. A much higher sensitivity of VSV infection to NH_4Cl was observed. Thus, a four-log

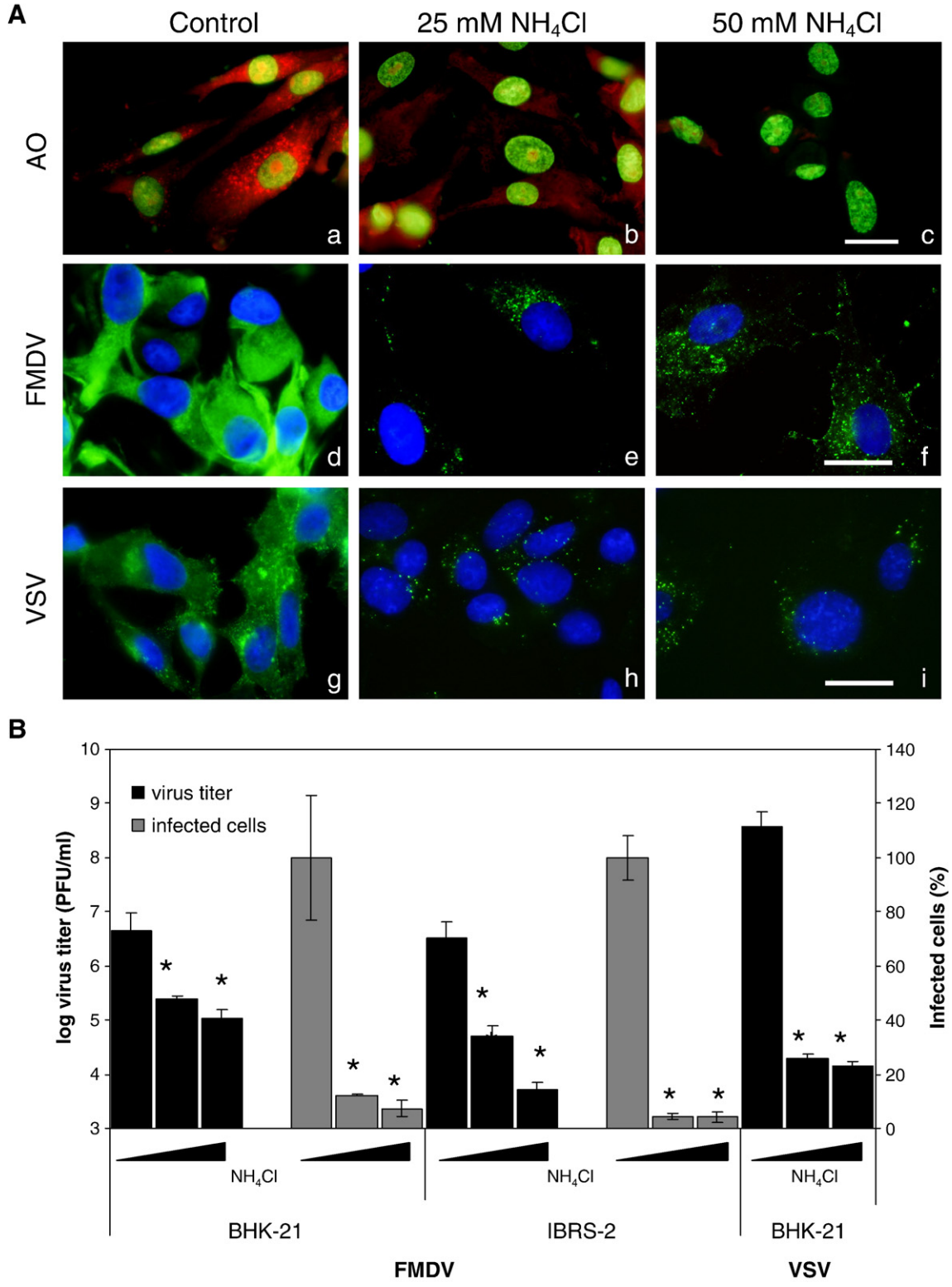


Fig. 4. Acidic pH requirement of FMDV and VSV infection. (A) Effect of different concentrations of NH_4Cl in FMDV and VSV infection of BHK-21 cells. (a) AO staining of control cells in which acidic organelles are stained in red and nuclei in green. (b) AO staining of cells treated 25 or (c) 50 mM NH_4Cl. (d–f) NH_4Cl treatment blocks FMDV infection. After 3 h of FMDV infection (MOI of 35), cells were fixed and processed for immunofluorescence using MAb 5C4. Untreated control cells (d) and cells treated with 25 or 50 mM NH_4Cl (e and f, respectively). (g–i) NH_4Cl treatment blocks VSV infection. Cells infected with VSV, as for FMDV, were fixed and processed for immunofluorescence using MAb I1. Scale bars: 20 μm. (B) NH_4Cl inhibits FMDV and VSV infection. Cells non-treated and treated with 25 or 50 mM NH_4Cl were infected with FMDV or VSV (MOI of 0.5). Total virus yield at 8 h PI was determined by plaque assay on BHK-21 cells. The number of infected cells expressing FMDV VP1 was determined, at 6 h PI, by immunofluorescence using MAb SD6, and the percentage of infected cells (relative to control untreated cells) is indicated. The means and SD from three independent experiments are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

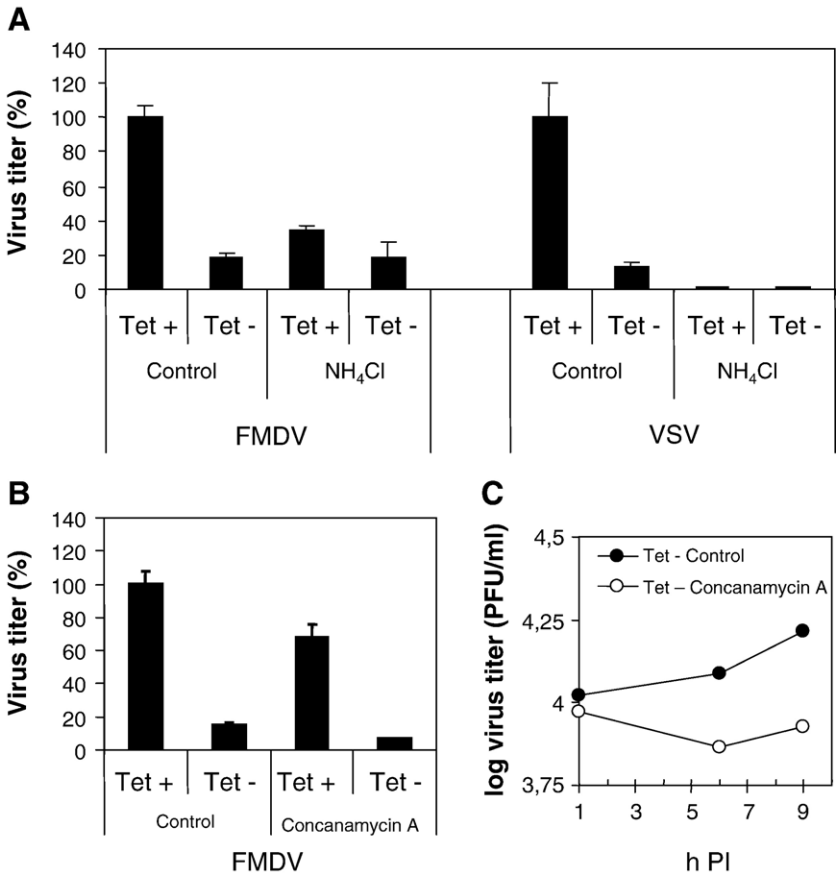


Fig. 5. Effect of endosomal acidification blockage of Tet+ and Tet− cells on FMDV and VSV internalization and infection. (A) Effect of NH_4Cl treatment. Tet+ and Tet− cells were treated with 25 mM NH_4Cl and then infected with FMDV or VSV (MOI of 0.5). Total virus yield, at 9 h PI, was determined by plaque assay in BHK-21 cells and is presented as a percentage, relative to values of Tet+ cells. The means and SD from three independent experiments are shown. (B) Effect of concanamycin A treatment. Tet+ and Tet− cells were treated with 1000 nM concanamycin A and total virus yield in infected cells was determined as in panel A. The means and SD from three independent experiments are shown. (C) Time course of FMDV growth on Tet− cells treated with 1000 nM concanamycin A. Tet− cells were infected with FMDV (MOI of 0.5) and at different times PI total virus yield was determined. The means of two independent experiments are shown; SD values were lower than 15%.

reduction in the virus yield was determined for either Tet+ or Tet− cells, infected with VSV in the presence of NH_4Cl.

These results could suggest that type C FMDV entering Tet− cells, which lack functional clathrin exploit alternative endocytic pathways that either led the virus to other compartment less affected by the acidification blockage exerted by NH_4Cl, or in which a pH decrease is not required for viral RNA release. This possibility was not supported by the results obtained using concanamycin A. With this drug, a limited, but significant, reduction in virus titer was observed at 9 h PI for Tet+ and Tet− cells (Fig. 5B). For Tet− cells, a limited increase with time in virus titer was observed, while such increase was not detectable, at different hours post-infection, in Tet− cells treated with concanamycin A (Fig. 5C). Thus, our results suggest that FMDV multiplication in Tet− cells requires acidic endosomes.

BHK-21 and IBRS-2 cells express caveolin-1

To evaluate whether caveola-mediated endocytosis could contribute to FMDV internalization, we first analyzed the expression of caveolin-1 in BHK-21 and IBRS-2 cells. Fig. 6A shows a western blot of cell extracts of BHK-21 and IBRS-2. The rabbit polyclonal antibody anti-caveolin 1 used showed that both cell lines expressed caveolin-1, being the level of expression detected higher in BHK-21 cells. As reported (Millan et al., 1997), no caveolin-1 expression was detected in Jurkat cells, used as control. Fig. 6B shows the immunofluorescence pattern of caveolin-1 in IBRS-2, which appeared as membrane-associated vesicles, corresponding to lipid-rafts and caveolae, as well as with a perinuclear distribution characteristic of a Golgi location, as reported (Kurzchalia et al., 1994). Similar results were obtained using BHK-21 cells (data not shown).

Role of cholesterol in FMDV endocytosis and virus production

Since BHK-21 and IBRS-2 cells express caveolin-1, we explored the effect on FMDV entry of drug treatments interfering with caveola-mediated endocytosis. Cholesterol is an important component of lipid-rafts, which are involved in caveola formation. Thus, we analyzed the effect on FMDV entry and infection progress of the cholesterol-sequestering compound nystatin that selectively disrupts membrane lipid-rafts, including those required for caveolae formation (Chen and

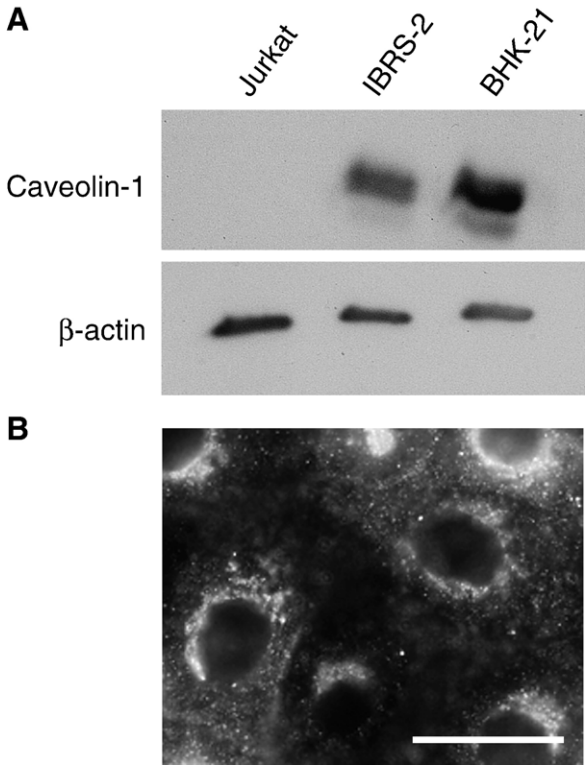


Fig. 6. Caveolin-1 expression in BHK-21 and IBRS-2 cells. (A) Western blot of BHK-21 and IBRS-2 cell extracts with anti-caveolin 1 antibody. Extracts of Jurkat cells that do not express caveolin-1 were included as control. Blotting to an anti-β-actin MAb was included as control of protein loading. (B) Immunofluorescence of IBRS-2 cells incubated with anti-caveolin 1 antibody and an Alexa Fluor 488-labeled secondary antibody. Scale bar: 20 μm.

Norkin, 1999), and of MβCD that depletes cholesterol from cell membranes (Sieczkarski and Whittaker, 2002a; Simons and Ikonen, 1997). A pre-treatment of cells with 25 μg/ml of nystatin for 1 h (70% and 82% cell viability for BHK-21 and IBRS-2, respectively) was chosen for these experiments. Fig. 7A shows that the internalization of labeled CTX, a lipid raft-ligand that can use different endocytic mechanisms (Torgersen et al., 2001), including a non-clathrin, non-caveolae pathway (Chinnapen et al., 2007), was, as expected, reduced in BHK-21 cells by the treatment with nystatin. Nevertheless, no effect was observed in the internalization of either FMDV or labeled TF (Fig. 7A).

Treatment of BHK-21 cells with 10 mM MβCD resulted in a severe depletion of the cholesterol at the cell membrane, as estimated by cell staining with the cholesterol-binding drug filipin. As reported (Keller and Simons, 1998), this treatment did not significantly alter the cholesterol found in intracellular membranes. A clear inhibition of CTX uptake was found in cells treated with MβCD. A similar effect was noticed in the internalization of FMDV, whose virions were preferentially detected at the surface of infected cells treated with MβCD (Fig. 7A). Similar results were observed in IBRS-2 cells treated with MβCD (Fig. S4). Uptake of TF was also affected by the MβCD treatment, confirming the requirement of cholesterol in the clathrin-mediated endocytosis, as previously reported (Rodal et al., 1999).

Then, we studied the effect of nystatin and MβCD on post-entry steps of viral cycle leading to productive infection. In fact, the results obtained with BHK-21 and IBRS-2 cells showed a good correlation with those observed on viral internalization. Thus, relative to control cells, no significant reductions in the percentage of infected cells expressing capsid protein VP1, or in the virus yield recovered from infected cultures treated with nystatin, were noticed (Fig. 7B). This lack of effect on the virus yield was also observed for VSV-infected cells. Conversely, depletion of cholesterol with MβCD in the two cell lines studied, resulted in a significant reduction of the FMDV-infected cells, which showed values of 63% and 40% for BHK-21 and IBRS-2, respectively. Similar reductions were observed in the virus production, which paralleled that found in VSV-infected cells (Fig. 7B).

Thus, while the reorganization of cholesterol induced by nystatin does not affect type C FMDV entry and progression, depletion of cholesterol from plasma membranes by MβCD decreases FMDV internalization and virus infection.

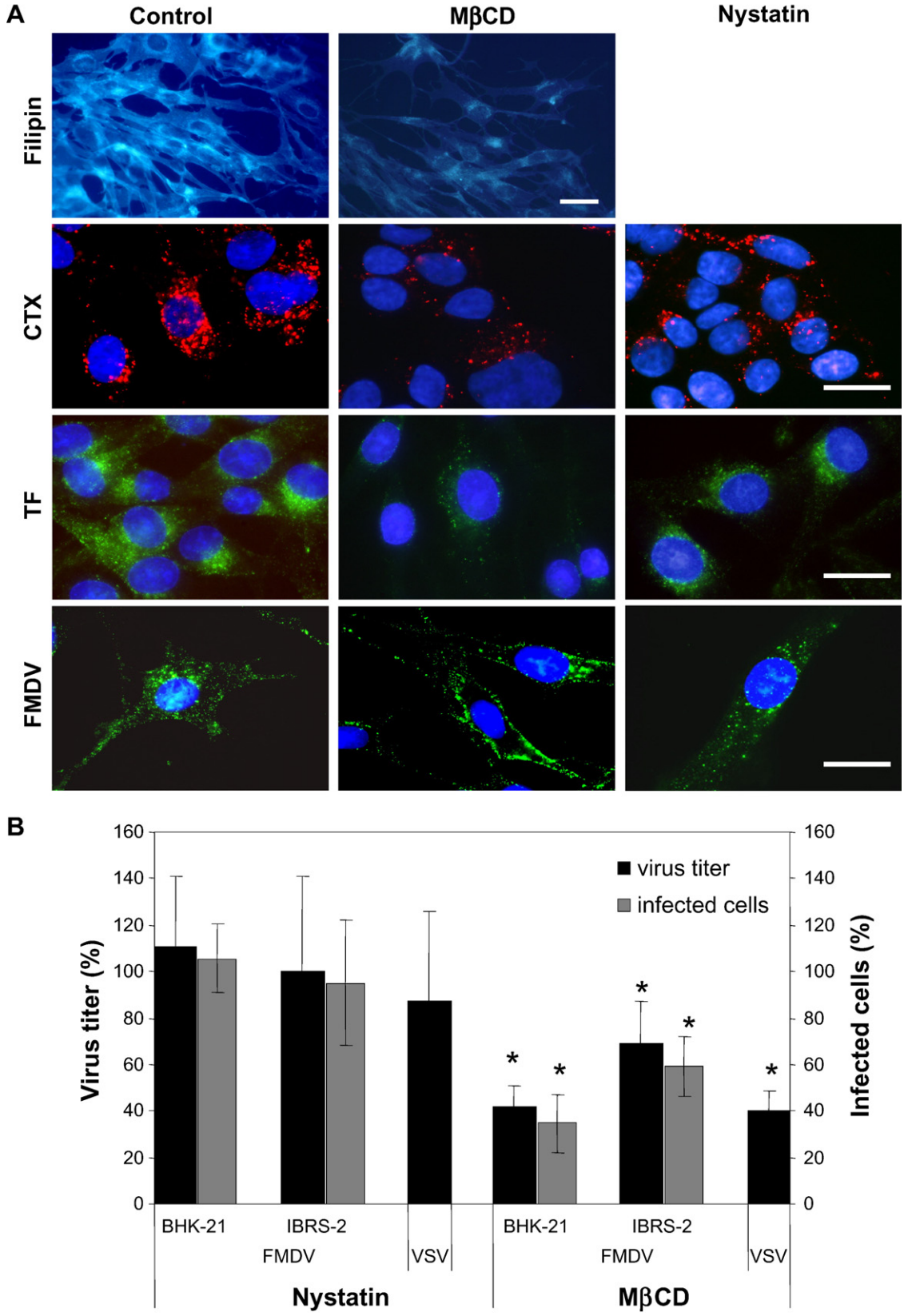
Discussion

Viruses that enter host cells by endocytosis are becoming interesting tools in studies of membrane traffic in animal cells. Recently, they have been used to characterize novel endocytic mechanisms that bypass the classical clathrin-mediated uptake processes (Damm et al., 2005; Pietiainen et al., 2004). The type C FMDV used in our study mainly enters established cell lines derived from porcine (IBRS-2) and hamster (BHK-21) cells by using a clathrin-dependent endocytosis, as indicated by uptake blockage of FMDV particles observed in immunofluorescence assays upon sucrose treatment. This blockage affects also the progression of virus infection, as indicated by the reductions in infected cells and virus yield in infections performed at low MOI to limit productive vital entries that could exploit pathways other than those more efficiently used by FMDV. These results extends previous observations on the entry requirements of FMDV isolates from different serotypes in cells expressing human $\alpha_v\beta_3$ and $\alpha_v\beta_6$ integrins (Berryman et al., 2005; O'Donnell et al., 2005), to the productive infection by serotype C FMDV of two widely used cell lines.

IBRS-2 cells express $\alpha_v\beta_8$ integrin (Burman et al., 2006), while BHK-21 have been reported to express $\alpha_v\beta_3$ (Chu and

Fig. 7. Role of cholesterol in FMDV entry and infection. (A) Effect of MβCD and nystatin on endocytosis pathways. BHK-21 cells treated with 10 mM MβCD or with 25 μg/ml of nystatin were incubated with Alexa Fluor 594-labeled CTX, Alexa Fluor 488 TF or infected with FMDV (MOI of 35) for 25 min, fixed and processed for immunofluorescence staining of VP1 using MAb 5C4 and an Alexa Fluor 488 secondary antibody. Control of cholesterol depletion after MβCD treatment was analyzed by filipin staining. Scale bars: 20 μm. (B) Analysis of virus infection after MβCD or nystatin treatment. Cells, incubated with nystatin or MβCD as described in panel A, were infected with either FMDV or VSV (MOI of 0.5). At 8 h PI, total virus yield was determined by plaque assay on BHK-21 cells. The number of infected cells expressing FMDV VP1 was determined, at 6 h PI, by immunofluorescence using MAb SD6. The percentages of virus titers and of infected cells are relative to those observed in untreated control cells. The means and SD from three independent experiments are shown.

Ng, 2004). The FMDV C-S8sc1 used in this study showed sequence identity in the capsid proteins to that published for the original isolate, which infected cultured cells via integrins receptors (Baranowski et al., 1998; Jackson et al., 2000). These, rule out the possibility of selection, during virus amplification, of the mutation reported to confer type C FMDV the capacity to



enter cultured cells via heparan sulphate (Baranowski et al., 1998). Since α_v integrins have been shown to be internalized into clathrin-coated pits and subsequently transferred to early endosomes (Berryman et al., 2005; Upla et al., 2004), the results reported here likely correspond to type C FMDV integrin-mediated entry and progression in BHK-21 and IBRS-2 cells. The lack of MAbs efficiently recognizing the integrins expressed in these cell lines (O'Donnell et al., 2005) has impaired the inclusion in our study of data from integrin internalization. We cannot exclude that the inhibition of clathrin-dependent endocytosis could alter expression of virus receptors at the cell surface leading to reduced virus binding and infection and this could contribute to the inhibitory effects found. However, FMDV appears attached at the cell membrane in cells treated with sucrose in our experiments as well as in those reported by (Berryman et al., 2005), suggesting that a major alteration in the expression of intergrins does not occur upon sucrose treatment. On the other hand, transferrin receptor (another ligand taken in by clathrin-dependent endocytosis), which co-localizes in the same endosomes with FMDV and its $\alpha_v\beta_6$ receptor (Berryman et al., 2005; O'Donnell et al., 2005), rather than being depleted, accumulates in the membrane surface of Tet− cells (Iversen et al., 2003). These results suggest that the inhibitions observed are mostly due to inhibition of uptake of surface-bound viruses from clathrin coated pits.

Interestingly, the results obtained with BHK21-tTA/anti-CHC show a direct, functional requirement of clathrin (CHC) for FMDV entry and multiplication. The dependence on CHC for infection of viruses using clathrin-mediated endocytosis has been previously reported utilizing different strategies. (Pelkmans et al., 2005; Sun et al., 2005; Wettey et al., 2002). However, to our knowledge, this is the first study that has utilized an inducible cell line against CHC for virus entry characterization. Other inducible cell lines, such as those expressing dominant negative forms of dynamin, have been revealed as valuable tools to uncover the entry route of different viruses (Chung et al., 2005; DeTulleo and Kirchhausen, 1998; Snyers et al., 2003). However, the negative mutant of dynamin can inhibit several types of endocytosis. It inhibits uptake from caveolae (Oh et al., 1998) as well as clathrin- and caveolae-independent endocytosis that can be dependent on dynamin (Lamaze et al., 2001).

In our experiments (Fig. 3D), a residual level of FMDV infection was observed when clathrin-mediated endocytosis was blocked by tetracycline removal in Tet− cells. Despite the low virus yield recovered from infected Tet− cells, no additive effect on this inhibition was observed upon NH_4Cl treatment, (Fig. 5A), while a reduction was observed in cells treated with concanamycin A (Figs. 5B, C). These observations suggest that type C FMDV entry in these cells, which lack functional clathrin, could exploit alternative endocytic pathways that led the virus to acidic compartments where viral RNA is released. Transit through early acidic endosomes for productive internalization can use endocytic pathways other than the clathrin-mediated, as reported for Influenza virus (Sieczkarski and Whittaker, 2002b) and diphtheria toxin (Skretting et al., 1999). However, we cannot exclude that the FMDV infection observed when clathrin is blocked could be due to some "leakiness" in the system rather than a true clathrin-independent entry phenomenon. In any case, further experiments are required to confirm this possibility.

The expression of caveolin-1 by BHK-21 and IBRS-2 cells has also allowed the analysis of the potential contribution of the caveolae-mediated internalization pathway in FMDV entry. As cholesterol is an important component of lipid-rafts, which are involved in caveolae formation, we analyzed the effect on FMDV entry and infection of nystatin, a sterol-binding drug that sequesters cholesterol, and of MβCD that depletes cholesterol from membranes (Sieczkarski and Whittaker, 2002a). The results obtained support that FMDV endocytosis in BHK-21 and IBRS-2 cells is not affected by the lack of lipid-rafts integrity reported to be induced by nystatin (Chen and Norkin, 1999) but depends on the presence of certain levels of cholesterol in the plasma membrane, probably as this molecule seems to be required for the formation of clathrin-coated vesicles (Rodal et al., 1999; Subtil et al., 1999), for the regulation of downstream stages (Peres et al., 2003) and for recruitment of clathrin vesicles machinery (Urs et al., 2005). Indeed, extraction of plasma membrane cholesterol with MβCD has been shown to inhibit clathrin-mediated endocytosis of human rhinovirus (Snyers et al., 2003) and a requirement of cholesterol, but not of lipids-rafts, has also been recently reported for poliovirus cell entry (Danthi and Chow, 2004). Previous studies, performed with a lower concentration of MβCD than that used in this study, showed that infection of SW480-$\alpha_v\beta_6$ cells with type O FMDV was not affected by MβCD treatment (Berryman et al., 2005). This discrepancy could be due to the different susceptibility to the drug of the cell types used, as reported in other viral models (Gilbert and Benjamin, 2000; Richterova et al., 2001).

Interestingly, the differences in the sensitivity to endosomal acidification inhibition by NH_4Cl or concanamycin A (Fig. 4 and Fig. S3) suggest that FMDV and VSV may uncoat into different classes of endosomes. Supporting this idea, VSV has been shown to deliver its genetic material to cytoplasm from late endosomes (Le Blanc et al., 2005), whereas FMDV has never been found in late endosomes or lysosomes (Berryman et al., 2005; O'Donnell et al., 2005). These differences in intracellular trafficking may reflect differential sorting from distinct populations of clathrin-coated pits involved in FMDV and VSV internalization. Such differences have been described for other ligands of the clathrin endocytic pathway that are sorted to populations of early endosomes that either maturate rapidly toward late endosomes, or that maturate much more slowly (Lakadamyali et al., 2006). However, these differences could not be revealed by treatments directly targeted to clathrin-coated pit endocytosis, as CHC depletion. These findings show the usefulness of performing comparative studies with different viruses in order to study in depth the multiple viral internalization pathways (Damm and Pelkmans, 2006; Pelkmans et al., 2005).

Materials and methods

Cells and viruses

BHK-21 cells (ATCC) and IBRS-2 (c-26) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) (Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml) and 20 mM L-glutamine. BHK21-tTA/anti-CHC cells (Iversen et al., 2003) were maintained in select DMEM supplemented with 10% FCS, 200 μg/ml geneticin (Gibco), 0.5 μg/ml puromycin (Sigma) and 2 μg/ml tetracycline (Sigma). For induction of CHC anti-sense RNA expression, cells were grown in select DMEM and tetracycline was removed 72 h prior to the experiments. FMDV isolate C-S8c1, a derivative of a type C field virus isolated in Santa Pau (Spain, 1970) by triple plaque purification (Sobrino et al., 1983), and VSV (Indiana serotype) were used in this study. Viral stocks were prepared by infecting at a multiplicity of infection (MOI) – defined as the plaque forming units of virus (PFU)/cell – of about 1, monolayers of BHK-21 cells, as described below. Viral RNA from C-S8c1 stock was amplified by RTR-PCR and used to determine the nucleotide sequences corresponding to the capsid proteins, as described (Nunez et al., 2001); the sequences obtained were as previously published (Toja et al., 1999).

Antibodies and reagents

FMDV VP1 and VSV G protein were detected using monoclonal antibodies (MAb) SD6 or 5C4 (Lea et al., 1994; Mateu et al., 1987) and MAb I1 (Lefrancois and Lyles, 1982), respectively. Rabbit polyclonal antibodies were used to detect caveolin-1 (BD Transduction Laboratories) and clathrin heavy chain (Sta. Cruz Biotechnology). MAb anti-β-actin AC-15 was from Sigma. Sheep anti-mouse IgG and donkey anti-rabbit IgG coupled to horseradish peroxidase were from Amersham. Goat anti-mouse IgG Alexa Fluor 488 or 594 labeled, as well as transferrin (TF) and cholera toxin (CTX) conjugated to Alexa Fluor 488 or 594, respectively, were from Molecular Probes. Nystatin, filipin III, methyl-β-cyclodextrin (MβCD), concanamycin A and bovine serum albumin (BSA) were purchased from Sigma. Sucrose and NH_4Cl were from Merck. Nystatin, filipin and concanamycin A were prepared in DMSO as 5 mg/ml, 2.5 mg/ml and 28.8 μM stock solutions, respectively. NH_4Cl (1 M) was dissolved in water and the rest of chemicals were directly prepared in DMEM.

Drug treatments

For endocytosis inhibition, cells grown on tissue culture plates or coverslips, were washed twice with DMEM and incubated with the corresponding drug for the appropriate time. Control cells were incubated in the same conditions in DMEM containing the solvent concentration used for each drug. To test the role of lipid-rafts and cholesterol in endocytosis, cells were pre-treated for 1 h with a nystatin concentration (25 μg/ml), which was selected after a viability assay, or by treatment with MβCD (10 mM) for 30 min, as previously reported (Keller and Simons, 1998). Nystatin was maintained during the whole infection period while MβCD was present only during pre-treatment. The role of clathrin in endocytosis was tested by a hypertonic shock induced by treating cells for 30 min with 0.45 M sucrose in DMEM before FMDV infection or fluorescent TF incubation, the later as control of the clathrin pathway impairment. To avoid cellular recovery, sucrose was maintained during the first hour of infection or during the TF internalization time. The lysosomotropic agent NH_4Cl was used to block endosomal acidification. Cells were pre-treated for 1 h with 25 or 50 mM NH_4Cl in DMEM with 25 mM HEPES pH 7.4 and the drug was maintained during the whole infection period to avoid cellular recovery. For concanamycin A treatment, cells were pre-treated 30 min and the drug was maintained during the first hour of infection, as reported (Berryman et al., 2005).

Infections and virus titration

Cells grown on coverslips or tissue culture plates were infected at different MOI with virus diluted in DMEM. After 1 h of adsorption, the inoculum was removed and fresh medium containing 5% FCS was added. Virus yield was determined by plaque assay on BHK-21 cell monolayers, as described (Sobrino et al., 1983). Briefly, cells were adsorbed with infection medium samples, the inoculum removed after 1 h and the infection allowed to proceed for 24 h in semisolid medium (0.5% agar, 1% FCS and 0.045 mg/ml DEAE-Dextran in DMEM) to determine virus titers (PFU/ml). Finally, the cells were fixed and stained with 0.3% crystal violet in 2% formaldehyde. To determine total virus production (intracellular and infection medium), plates were subjected to three freeze–thaw cycles and the virus titer determined as described above. When performing infections for western blot analyses or to determine FMDV virus yield after endocytosis inhibition, extracellular virions were acid inactivated after the first hour of infection by washing with PBS pH 6.0, followed by two washes with DMEM to neutralize extracellular pH. For plaque reduction assays, cell monolayers were infected with 50–100 viral PFU, and the PFU developed in semisolid medium were quantified as described above.

Transferrin and cholera toxin internalization

For TF endocytosis assays, cells grown on coverslips, were washed twice with DMEM and were incubated with 10 μg/ml TF-Alexa Fluor 488 prepared in DMEM containing 0.5% BSA. After 5 min of internalization at 37 °C, cells were washed with 200 mM acetic acid and 200 mM NaCl and sequentially with 150 mM NaCl, 20 mM HEPES pH 7.4, 10 mM glucose, 5 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ to eliminate extracellular TF (Lukacs et al., 1997). Finally, the cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS. In the case of CTX endocytosis assays, cells were incubated 30 min with CTX-Alexa Fluor 594 (8 μg/ml) in DMEM containing 0.5% BSA, washed twice with PBS and fixed. Aldehyde fluorescence

was quenched by incubating cells 15 min with 1 M glycine in PBS.

Immunofluorescence

Cells grown on coverslips were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 20 min, washed with PBS and permeabilized with BPTG (1% BSA, 0.1% Triton-X 100, 1 M glycine in PBS) for 15 min at room temperature (RT). Primary antibodies prepared in 1% BSA in PBS were incubated for 1 h at RT. Cells were washed with PBS, stained with the corresponding fluorescent antibody for 30 min at RT and then washed with PBS. Cell nuclei were counter-stained with 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole) in PBS for 5 min, rinsed with PBS and mounted in Fluoromount G (Southern Biotech Assoc., USA). Samples were observed using an Olympus BX61 epi-fluorescence microscope coupled to a digital camera DP70 (Olympus). Images were acquired using Olympus DP controller software and processed using Adobe Photoshop 7.0 (Adobe Systems Inc.).

Detection of infected cells

Monolayers of BHK-21 or IBRS-2 cells grown on glass coverslips were infected, fixed at 6 h post-infection (PI) and processed for immunofluorescence using MAbs SD6 and I1 to detect FMDV or VSV, respectively. For quantification of infected cells, a minimum of 300 cells per coverslip were scored (three coverslips).

Acridine orange vital staining

Fluorescent labeling of acidic organelles was performed using acridine orange (AO) vital staining, as reported (Al-Younes et al., 1999). AO fluorescence is red when it protonates and accumulates in acidic vesicles. Briefly, cells treated with NH_4Cl as described above were incubated in fresh medium, containing or not NH_4Cl and 5 μg/ml AO for 10 min at 37 °C, washed three times with the same media without AO and observed under fluorescence microscope using a filter set that permits simultaneous observation of green and red fluorescence.

Filipin staining

Cholesterol levels in cells treated with MβCD were estimated by filipin staining, as previously described (Keller and Simons, 1998). Briefly, cells were fixed on ice in 4% paraformaldehyde, stained 15 min at RT with 125 μg/ml filipin in PBS, washed twice with PBS for 15 min and mounted in Fluoromount G.

Western blot

Cells were lysed on ice in lysis buffer (10 mM EGTA, 2.5 mM $MgCl_2$, 1% NP-40, 20 mM HEPES pH 7.4), collected with a cell scraper and sonicated. Equal amounts of protein, estimated by Bradford, were mixed with Laemmli sample buffer. After SDS–PAGE electrophoresis proteins were transferred onto nitrocellulose membranes and incubated with specific antibodies, then with HRP-coupled anti-mouse or anti-rabbit antibodies and subsequently developed using western lighting chemiluminescence reagent (Perkin Elmer), as described (Garcia-Briones et al., 2006). Relative CHC levels were determined by image analysis using a Typhoon 9410 scanner and ImageQuant TL software (Amersham).

Data analysis

Analysis of the variance (ANOVA) using *F* Fischer–Snedecor distribution was performed with statistical package SPSS v.12 (SPSS Inc.) for Windows. Differences were considered statistically significant at $p<0.05$ and are indicated by an asterisk in the figures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.07.021.

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