Bovine Viral Diarrhea Virus Entry Is Dependent on Clathrin-Mediated Endocytosis

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Cellular mechanisms of bovine viral diarrhea virus (BVDV) entry in MDBK cells were investigated. Chloroquine, bafilomycin A1, or ammonium chloride inhibited BVDV infection, indicating that an acidic endosomal pH is required for BVDV entry. The tyrosine kinase inhibitor genistein partially inhibited BVDV infection at a postentry step, whereas BVDV entry was strongly inhibited by chlorpromazine or by the overexpression of a dominant-negative form of EPS15, a protein essential for the formation of clathrin-coated vesicles at the plasma membrane. Together, these data indicate that BVDV infection requires an active clathrin-dependent endocytic pathway.

Bovine viral diarrhea virus (BVDV) is a small, enveloped, positive-stranded RNA virus which is the etiological agent of a variety of pathologies in cattle, including fatal mucosal disease (21, 31). BVDV is classified in the *Pestivirus* genus within the *Flaviviridae* family, which also contains hepatitis C virus (HCV) and viruses of the *Flavivirus* genus (21). Little is known about cellular mechanisms leading to the entry of BVDV and other pestiviruses. Their binding to target cells involves envelope glycoproteins E^{ns} and E2 (16, 20, 27, 33) through interactions with glycosaminoglycans (17, 18) and membrane proteins (28, 35), respectively. BVDV receptors include CD46 (22) and low-density-lipoprotein receptor (1). Because of their similarity to flaviviruses, pestiviruses are thought to enter target cells by receptor-mediated endocytosis and acid-triggered fusion of their envelope with an endosomal membrane (1, 22). However, BVDV is also known to be resistant to acidic treatments (9, 19), a condition often found in viruses with pH-independent entry.

In order to assess if BVDV entry is pH-dependent, we first sought to determine if BVDV infection indeed requires low endosomal pH. The importance of endosome acidification was studied with chloroquine and ammonium chloride, two lysosomotropic agents, and with bafilomycin A1, a specific inhibitor of endosomal proton-ATP pumps. MDBK cells were preincubated for 30 min with inhibitors, infected for 1 h at 37°C with BVDV (NADL strain) (23), and cultured for 15 h in the presence of the inhibitors. The virus was diluted such that 30 to 40% of the cells were infected in control experiments with no inhibitor. The infected cells were detected by indirect immunofluorescence microscopy by using a monoclonal antibody to NS3 (5). The nuclei were stained with Hoechst dye. The infections were scored as the ratio of infected cells to total cells. For comparison, we used bovine herpesvirus 1 (BHV-1), which is known to enter cells by a pH-independent mechanism (34). Each drug inhibited BVDV infection in a dose-dependent

manner (Fig. 1). In contrast, BHV-1 infection was not inhibited by chloroquine or bafilomycin A1, and ammonium chloride treatment resulted in a partial inhibition of BHV-1 infection. To verify that these drugs interfered with BVDV entry, we asked whether they could act on an early step of the infection. Bafilomycin was added during early or late steps of infection. The drug strongly inhibited BVDV infection when present during the infection and up to 4 h postinfection but was without effect when added later (Fig. 1). Similar results were obtained with chloroquine (data not shown). Taken together, the results obtained with these inhibitors indicate that BVDV infection is sensitive to the low pH of endosomes. Similar results were recently reported by others (12, 19).

The pH-dependent entry of BVDV suggests that the virus is internalized from the cell surface by receptor-mediated endocytosis and reaches an endosomal compartment, where the fusion occurs. Two well-defined endocytic pathways appear to be clathrin-mediated endocytosis and caveolae internalization (25, 29). To determine if BVDV enters cells through a clathrin-mediated or a caveolae-mediated pathway, we first tested the effects of chlorpromazine (32) and genistein (8, 24), respectively. For comparison, the effects of these inhibitors were also assessed on the infection of Sindbis virus, which enters by clathrin-mediated endocytosis (6). Chlorpromazine inhibited BVDV infection in a dose-dependent manner (Fig. 2A). The inhibition was almost complete at a concentration of 10 mM, which is known to block clathrin-coated pit assembly at the plasma membrane (32). As expected, Sindbis virus infection was inhibited by chlorpromazine and BHV-1 infection was not affected, suggesting that the observed effects were not due to the toxicity of the drug (Fig. 2A).

Genistein also inhibited BVDV infection in a dose-dependent manner (Fig. 2B). We observed about 50% inhibition with 50 to 100 µg/ml genistein. Similar concentrations of genistein inhibit simian virus 40 internalization (8) but are without effect on influenza virus infection (30). Sindbis virus infection was not affected in the presence of 50 µg/ml genistein (Fig. 2B). This suggests that the inhibition of BVDV infection by genistein was not due to toxicity on MDBK cells. Taken to-

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FIG. 1. BVDV infection requires an acidic endosomal pH. (A) MDBK cells were preincubated for 30 min with 100 nM bafilomycin A1, 100 μM chloroquine, or 5 mM ammonium chloride or without drugs (control) and subsequently infected with BVDV at a multiplicity of infection of 0.3 for 1 h. Cells were rinsed three times and cultured for 15 h. The inhibitors were present throughout the experiment. Cells were fixed and processed for immunofluorescence by using an antibody to NS3. (B through D) MDBK cells were infected with BVDV (black bars) or BHV-1 (gray bars) in the presence of different concentrations of bafilomycin A1, chloroquine, or ammonium chloride. (E) MDBK cells were infected with BVDV (black bars) or BHV-1 (gray bars) and treated with 100 nM bafilomycin A1 from the preincubation up to 4 h postinfection (0–4 h) or up to 15 h postinfection (0–15 h) or infected in the absence of inhibitor and treated with the inhibitor from 4 h postinfection up to 15 h postinfection (4–15 h). Infected cells were detected by immunofluorescence. Total cells were detected by the staining of nuclei with Hoechst dye. The infectivities were quantified as the ratios of infected cell numbers to total cell numbers and are expressed as percentages of infectivity in control cells. Values are means from two independent experiments.

gether, these data show that BVDV infection is sensitive to both chlorpromazine and genistein.

We next verified if these drugs inhibit an early step of BVDV infection. As shown in Fig. 2C, the inhibition of BVDV infection was strong when chlorpromazine was added to the cells for only 4 h at the beginning of the infection but was very weak

when the drug was added onto the cells four hours after infection. This action of chlorpromazine during early steps of BVDV infection is consistent with an inhibition of endocytosis. On the other hand, the inhibition by genistein was weaker when the inhibitor was added to the cells during the early steps than during the late steps of infection (Fig. 2C). Therefore, it is unlikely that genistein had an effect on BVDV endocytosis. These results indicate that BVDV entry is inhibited by chlorpromazine, suggesting that it might proceed through clathrin-mediated endocytosis.

This finding is consistent with the recent report that a dominant-negative form of dynamin blocks BVDV infection (19). However, dynamin regulates clathrin-mediated endocytosis, as well as other clathrin-independent pathways of internalization (14). Likewise, none of the drugs that we used are specific endocytosis inhibitors. To confirm that clathrin-mediated endocytosis was involved in BVDV entry, we made use of EΔ95-295, a dominant-negative form of EPS15, which specifically interferes with clathrin-coated vesicle formation at the plasma membrane (3). A recombinant adenoviral vector (rAd:EΔ95-295) was generated to express this mutant, and its functionality was verified on MDBK cells (data not shown). For a control, we used DIIIΔ2, another form of EPS15 with no dominant-negative effect on clathrin-mediated endocytosis (4). MDBK cells were transduced with rAd:EΔ95-295 or rAd:DIIIΔ2 and subsequently infected with BVDV or control viruses 24 h later. The number of infected cells was dramatically reduced in cells transduced with rAd:EΔ95-295, compared to cells transduced with rAd:DIIIΔ2 (Fig. 3A). The infection was scored separately in cells expressing low, moderate, or high levels of EΔ95-295. As expected, EΔ95-295 did not inhibit BHV-1 infection. In contrast, both BVDV and Sindbis virus infections were affected and the inhibition was positively correlated to EΔ95-295 expression levels (Fig. 3B). These data confirm that a functional clathrin-mediated pathway of endocytosis is required for BVDV infection.

A clathrin-mediated entry route has been reported previously for flaviviruses. Both electron microscopy studies and EΔ95-295-mediated inhibition indicated that West Nile Virus enters cells through clathrin-coated vesicles (7, 11). Since clathrin-coated vesicles deliver their cargo in acidic early endosomes, this is consistent with numerous studies which have shown that the envelope glycoprotein E of flaviviruses undergoes a conformational transition under acidic conditions from a native dimeric form to a fusogenic trimeric form (13). Such an acid-triggered conformational transition has not been reported at the present time for the envelope proteins of BVDV or other pestiviruses. In contrast, BVDV is resistant to acidic treatments (9, 19). Such a discrepancy between pH-dependent entry and resistance of the virion to acidic treatments has also been observed for vesicular stomatitis virus (26). It has recently been suggested that BVDV envelope glycoproteins must be primed by disulfide bridge reduction in the endocytotic pathway before an acid-sensitive fusogenic conformation can be reached (19).

In conclusion, our study suggests that, like flaviviruses, pestiviruses enter cells through clathrin-mediated endocytosis and fusion from within an acidic endosomal compartment. It would be interesting to determine if this pathway of endocytosis is also used by other pestiviruses and by HCV and other non-

FIG. 2. Effects of chlorpromazine and genistein on BVDV infection. MDBK cells were infected with BVDV (black bars), Sindbis virus (SIN; white bars), or BHV-1 (gray bars) in the presence of the indicated concentrations of chlorpromazine (A) or genistein (B). The cells were preincubated with the inhibitor for 30 min, and the inhibitor was included in culture medium throughout the experiment. Cells were fixed at 12 h, 4.5 h, or 8 h postinfection, and infection was detected by immunofluorescence. In order to facilitate the immunofluorescent detection of Sindbis virus infection, we used a recombinant Sindbis virus driving the expression of the envelope protein E1 of HCV in infected cells (10). (C) MDBK cells were infected and treated with 10 mM chlorpromazine (black bars) or 50 μg/ml of genistein (gray bars) from the preincubation up to 4 h postinfection (0–4 h) or up to 20 h postinfection (0–20 h) or infected in the absence of inhibitor and treated with the inhibitor from 4 h postinfection up to 20 h postinfection (4–20 h). The infectivities are expressed as percentages of infectivity in mock-treated controls. The data shown are the means and standard deviations from two independent experiments performed in triplicate (A and B) or in duplicate (C).

classified viruses of the *Flaviviridae* family, such as GB viruses. Recent data suggest that retroviral particles pseudotyped with HCV envelope glycoproteins E1 and E2 are sensitive to agents that neutralize the pH of endosomes (2, 15), but data on clathrin requirement have not yet been reported.

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FIG. 3. BVDV infection is inhibited in cells expressing EΔ95-295. (A) MDBK cells were transduced with either rAd:EΔ95-295 (dominant negative of EPS15) or rAd:DIIIΔ2 (control). Twenty-four hours later, transduced cells were infected with BVDV. Twelve hours after infection, cells were fixed and processed for immunofluorescence by using an antibody to NS3 followed by an Alexa546-coupled secondary antibody (BVDV). EΔ95-295- and DIIIΔ2-expressing cells were detected by the fluorescence of green fluorescent protein tags fused to EPS15 constructs (GFP). Arrowheads point to cells expressing EPS15 control construct that were infected with BVDV. (B) MDBK cells were transduced with either rAd:EΔ95-295 or rAd:DIIIΔ2 and subsequently infected with BVDV (black bars), Sindbis virus (SIN; white bars), or BHV-1 (gray bars) at a multiplicity of infection of about 0.3. Cells were fixed at 12 h, 4.5 h, or 8 h postinfection, and infection was detected by immunofluorescence. EΔ95-295- and DIIIΔ2-expressing cells were detected by green fluorescent protein fluorescence. The numbers of infected cells were scored according to the level of EΔ95-295 expression. The infectivities are expressed as percentages of infectivity in untransduced cells. The data shown are the means and standard deviations from three independent experiments.

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