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Porcine circovirus 2 infection of epithelial cells is clathrin-, caveolae- and dynamin-independent, actin and Rho-GTPase-mediated, and enhanced by cholesterol depletion

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ABSTRACT

Epithelial cells are the major *in vivo* target cells for porcine circovirus type 2 (PCV2). Although these cells are used for most studies of PCV2 gene expression and, little is known on PCV2 entry, attachment and internalization, in epithelial cells. PCV2 attachment to epithelial cells occurred rapidly and in a time-dependent manner. In contrast to attachment, internalization was slow. Immunofluorescent stainings revealed that during internalization, PCV2 co-localized with clathrin, but not caveolin. Blocking clathrin-mediated endocytosis increased instead of decreased the number of PCV2-infected cells by threefold, suggesting that it does not represent the main internalization pathway leading to a full replication. Further analysis with different inhibitors revealed that also macropinocytosis, dynamin-dependent internalization and membrane cholesterol play no role in PCV2 entry that leads to infection. Inhibition of small GTPases with *Clostridium difficile* toxin B reduced the number of PCV2-infected PK-15, SK and STs to 63 ± 25%, 47 ± 21% and 14 ± 6%, respectively. Finally, inhibiting actin polymerization also blocked PCV2 infection, showing the need for actin during PCV2 infection. Together, these data indicate that a dynamin- and cholesterol-independent, but actin- and small GTPase-dependent pathway, allows PCV2 internalization in epithelial cells that leads to infection and that clathrin-mediated PCV2 internalization in epithelial cells is not followed by a full replication.

1. Introduction

Porcine circoviruses are unenveloped viruses classified into the genus *Circovirus* of the family *Circoviridae* (Pringle, 1999; Tischer et al., 1982; Todd et al., 2005). Porcine circovirus type 2 (PCV2) genome is encapsidated by a single capsid protein of approximately 30 kDa (Nawagitgul et al., 2000). Replicase proteins (Rep and Rep′) that are essential for PCV2 DNA replication are encoded by open reading frame 1 (ORF1) present on the encapsidated viral DNA strand (positive sense) (Mankertz et al., 1998). The capsid protein is encoded by ORF2 on the antigenomic complementary DNA strand (negative sense) of the double-stranded replication intermediate synthesized in the host cell (Nawagitgul et al., 2000). ORF3 on the antigenomic complementary DNA strand encodes a 105 aa protein that has been reported to be involved in PCV2-induced apoptosis (Liu et al., 2005). PCV2 replicates its DNA genome via the rolling-circle replication mechanism (Cheung, 2004a,b).

In epithelial cells and cells of the porcine monocyte/macrophage lineage, PCV2 binds to heparan sulfate and chondroitin sulfate B glycosaminoglycans (GAG) (Misinzo et al., 2006). In porcine monocyte/macrophage cells, PCV2 binds quickly to all cells, while in contrast viruses are internalized slowly via clathrin-mediated endocytosis in only some of the cells (Misinzo et al., 2005). In dendritic cells, PCV2 is also internalized via clathrin-mediated endocytosis (Vincent et al., 2005). Although a lot is known on PCV2 gene expression and genome replication in porcine epithelial cells, little information is available on the virus replication steps preceding gene expression including PCV2 binding and internalization in this cell type. Apart from clathrin-mediated endocytosis, there are other pathways that may internalize molecules from the surface of eukaryotic cells and that are commonly hijacked by viruses to enter the host cell (Conner and Schmid, 2003; Mayor and Pagano, 2007). These other pathways include macropinocytosis, caveolin-mediated endocytosis and clathrin- and caveolin-independent pathways (CCIP) (Conner and Schmid, 2003; Marsh and Helenius, 2006; Mayor and Pagano, 2007). CCIP are further classified based on either the requirement for a dynamin-mediated scission mechanism, i.e. dynamin-dependent CCIP and dynamin-independent CCIP or involvement of small GTPases, cyclin-dependent cyclase

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42 (cdc42)-, Ras-homologous (Rho) GTPase A (RhoA)- or ADP-ribosylation factor 6 (Arf6)-regulated CCIP (Mayor and Pagano, 2007).

Some viruses are internalized in the same cell type via multiple endocytic pathways, e.g. human adenovirus type 2 and 5 can be internalized both via clathrin-mediated endocytosis and macropinocytosis (Meier and Greber, 2004); influenza virus can be internalized via clathrin-mediated endocytosis (Marsh and McMahon, 1999) and CCIP (Rust et al., 2004; Sieczkarski and Whittaker, 2002). Virus internalization pathways for some viruses, e.g. Epstein–Barr virus, human immunodeficiency virus and Semliki forest virus, can be cell type-dependent (Marsh and Bron, 1997; Miller and Hutt-Fletcher, 1992; Vidricaire and Tremblay, 2007). Because virus internalization can be cell type-dependent, data obtained from PCV2 internalization in porcine monocyte/macrophage and dendritic cells cannot be extrapolated to epithelial cells.

This study was carried out to analyze PCV2 binding and internalization kinetics and to investigate internalization pathways in porcine kidney (PK-15), swine kidney (SK) and swine testicle (ST) epithelial cells.

2. Materials and methods

2.1. Cells, virus, PCV2 virus-like particles (VLP) and reagents

PK-15, SK and ST epithelial cell lines free from porcine circoviruses were used in this study. Epithelial cells were seeded at 2×10^5 cells/ml and maintained at 37 °C in RPMI-1640 (Invitrogen, Grand Island, USA) containing 10% fetal bovine serum (FBS; Invitrogen), 0.3 mg/ml L-glutamine (BDH Chemicals Ltd., Poole, England), 1% non-essential amino acids (100×; Invitrogen), 1 mM sodium pyruvate (Invitrogen), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. Experiments were performed 24 h post-seeding in semi-confluent cell cultures and cells were maintained in a humidified 5% CO_2 incubator.

Epithelial cells were inoculated with the prototype PCV2 strain Stoon-1010 (5.3 $\log_{10}$ $TCID_{50}$, 20th passage in PK-15 cells) at a multiplicity of infection (m.o.i) of 0.3 for 1 h at 37 °C.

Recombinant PCV2 VLP were used in this study to investigate PCV2 binding and internalization kinetics, and in co-localization experiments with clathrin and caveolin-1. The use of PCV2 VLP in studying PCV2 binding to and internalization by target cells has been described and optimized in the previous studies (Misinzo et al., 2005, 2006).

Amiloride, chlorpromazine, *Clostridium difficile* toxin B, cytochalasin D, latrunculin B, filipin and methyl-β-cyclodextrin were obtained from Sigma (Bornem, Belgium). Myristoylated dynamin inhibitory peptide (DIP) was obtained from Tocris bioscience (Bristol, UK).

2.2. Kinetics of PCV2 binding to epithelial cells

Previously characterized PCV2 VLP (Misinzo et al., 2005) were used to establish the binding kinetics of PCV2 on PK-15, SK and ST epithelial cells. Epithelial cells were washed with ice-cold RPMI-1640 and chilled on ice for 30 min before ice-cold PCV2 VLP were added to cells for 1, 5, 10, 15, 30 and 60 min at 4 °C. Unbound PCV2 VLP were washed off using ice-cold RPMI-1640 before epithelial cells were fixed using 3% (w/v) paraformaldehyde dissolved in phosphate-buffered saline with calcium and magnesium (PBS+) at room temperature for 10 min. An immunofluorescence staining for bound PCV2 VLP was performed using anti-PCV2 capsid-specific monoclonal antibody F190 (McNeilly et al., 2001) followed by FITC-conjugated goat anti-mouse IgG (Invitrogen), each for 1 h at 37 °C. Finally, epithelial cells were mounted and a series of *z*-sections from the top to the bottom of epithelial cells from 10 randomly selected fields were acquired using a Leica TCS SP2 laser-scanning spectral confocal system linked to a Leica DM/IRB inverted microscope (Leica Microsystems, GmbH, Heidelberg, Germany). A merge of a series of *z*-section images per field (3543 μm^2) was performed to create a single overlay image. The total fluorescence area of attached PCV2 VLP per field was then calculated using the image analysis software SigmaScan Pro 5.0 as previously described (Misinzo et al., 2005, 2006).

2.3. Kinetics of PCV2 internalization into epithelial cells

PK-15 cells were chilled on ice for 30 min and washed with ice-cold RPMI-1640. PCV2 VLP in ice-cold RPMI-1640 were then incubated with epithelial cells at 4 °C for 1 h to allow binding onto the cells. Unbound PCV2 VLP were washed off and medium without FBS was added. Epithelial cells were shifted to a 37 °C humidified 5% CO_2 incubator. At 0, 5, 15, 60, 120 and 180 min after shifting the cells to 37 °C, cells were fixed in 3% (w/v) paraformaldehyde in PBS+. A double immunofluorescence staining was performed in order to distinguish bound from internalized PCV2 VLP. Bound PCV2 VLP were stained using anti-PCV2 capsid-specific monoclonal antibody F190 followed by FITC-conjugated goat anti-mouse IgG. Cells were subsequently washed with PBS+ and permeabilized with Triton X-100 (0.1% in PBS+) for 2 min at room temperature. After permeabilization, all PCV2 VLP were identified using the same monoclonal antibody F190 followed by Texas Red-conjugated goat anti-mouse IgG (Invitrogen). Finally, cells were mounted and analysis of PCV2 VLP internalization was done on images acquired using fluorescence confocal microscopy.

2.4. The effect of different inhibitors of endocytosis on PCV2 infection of epithelial cells

Different chemical agents known to inhibit endocytosis were used in this study in order to investigate the internalization pathway of PCV2 in epithelial cells. Their specific mode of action is indicated in Table 1. PK-15, SK and ST cells were washed and incubated for 2 h in a 5% CO_2 incubator at 37 °C with or without inhibitors of endocytosis diluted in cell culture medium without FBS. Afterwards, epithelial cells were inoculated with PCV2 (m.o.i. 0.3) for 1 h at 37 °C and 5% CO_2 in the presence or absence of inhibitors. The viral inoculum was washed off and epithelial cells were further incubated in cell culture medium containing 10% FBS with or without inhibitors and at 37 °C with 5% CO_2. At 24-h post-inoculation (hpi), the inhibitors were replaced with cell culture medium containing 10% FBS without inhibitors. In epithelial cells, treated with or without DIP, a similar protocol was used except that cells were incubated with the inhibitor in cell culture medium without FBS until 12 hpi, after which the cells were cultured in cell culture medium containing 10% FBS without the inhibitor until fixation. The highest concentration of each of the inhibitors did not affect the viability of cells under the present conditions. Cell viability was determined by propidium iodide staining as previously described (Misinzo et al., 2005). Untreated and treated cells were fixed at 36 hpi in methanol at −20 °C for 10 min. PCV2-infected epithelial cells were identified using an immunoperoxidase monolayer assay (IPMA) as described (Labarque et al., 2000). PCV2-infected epithelial cells were counted by examination under an Olympus light microscope (Olympus Optical Co., Hamburg, Germany). The number of infected cells per well of a 96-well cell culture plate (Nunc, Roskilde, Denmark) in untreated cells was used as a reference and the number of infected cells in treated cells was expressed as a relative percentage of