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Clathrin- and serine proteases-dependent uptake of porcine epidemic diarrhea virus into Vero cells

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV), a member of the genus *Alphacoronavirus*, is a causative agent of porcine enteric disease characterized by acute watery diarrhea and dehydration in sucking piglet. Similar to other coronaviruses, PEDV spike protein mediates its cell entry by binding to cellular receptors and inducing membrane fusion between viral envelopes and cellular membranes. However, the entry mechanism of PEDV is not studied. Here, we determined the entry mechanism of PEDV into Vero cells. Our data confirmed that PEDV entry followed clathrin-mediated endocytosis independence of caveolae-coated pit assembly. The internalized PEDV was co-localized with the clathrin-mediated endocytic marker, but not with the caveolae-mediated endocytic marker. In addition, cells treated with lysosomotropic agents and serine protease inhibitors were resistant to PEDV. Our data revealed that PEDV entry followed clathrin-mediated endocytosis and was dependent on a low pH and serine proteolysis for successful entry into cells.

1. Introduction

Infection of enveloped viruses is initiated by binding of surface proteins with specific receptor(s) on the surface of the cell membrane, which leads to internalization of the virus into cells. The second step of infection following virus attachment is the uncoating of the viral genome into the cytoplasm after the viral envelope has fused with the host membrane. There are two major routes for enveloped viruses to enter host cells; the non-endosomal and the endosomal pathways (Pelkmans and Helenius, 2003; Smith and Helenius, 2004). Both pathways require the release of the viral genome by fusion of the viral envelope with the respective target membrane of the host cells such as the plasma or endosomal membrane, respectively (Matlin et al., 1981). In the non-endosomal pathway, the viral envelope directly fuses with the plasma membrane. Membrane fusion is mediated by a conformational change of the viral glycoprotein, which is induced by its interaction with the corresponding receptor on the host cell surface and/or proteolytic processing (Blumenthal et al., 2002). The endocytic pathway can further differentiate into two well-characterized pathways; those acting via the clathrin-coated pit and the caveolae-mediated lipid raft (Brodsky et al., 2001; Pelkmans and Helenius, 2002). After internalization, viruses require a low-pH environment in the endosome to trigger conformational changes in the viral glycoproteins. The acidic pH environment is also important for proteolytic activation of viral glycoproteins by endosomal proteases (Qiu et al., 2006; Simmons et al., 2005).

The porcine epidemic diarrhea virus (PEDV) is classified as *Alphacoronavirus* together with transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV), and human coronavirus 229E (hCoV-229E). PEDV causes an acute watery diarrhea in suckling piglets, which results in approximately 50% mortality among suckling piglets and reduces the weight among fattening pigs (Debouck and Pensaert, 1980). Porcine epidemic diarrhea (PED) is first recognized in pigs in the United Kingdoms in 1971 (Wood, 1977). Although no evidence of PED is currently reported from Canada, similar coronavirus-like particles were reported from herds in Quebec in 1980 (Turgeon et al., 1980). Since then, outbreaks of PED have been documented in many European and Asian countries such as Czech Republic, Hungary, Korea, the Philippines, China, Italy, Thailand, Germany, Spain, and Japan (Song and Park, 2012). Recently, PEDV is spreading rapidly in swine farms in the United States, resulting in high mortality in piglets in more than 17 states (Mole, 2013).

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As typical for the *Alphacoronavirus*, the PEDV spike (S) protein encounters virus entry into host cells by interacting with its receptor, porcine aminopeptidase N (APN), in porcine enterocytes and by mediating membrane fusion with host cell membranes (Li et al., 2007; Oh et al., 2003). Upon receptor binding, several coronaviruses in *Alphacoronavirus* enter cells via endocytosis. For example, extensive studies on hCoV-229E have shown that upon binding with the human APN receptor, it is taken up in lipid rafts and enters via caveolae-dependent endocytosis (Nomura et al., 2004). Inside the endosome, cellular proteases that are active in a low-pH environment facilitate membrane fusion (Kawase et al., 2009). Similarly, TGEV binds to porcine APN (Weingartl and Derbyshire, 1994), and has been shown to enter MDCK cells over-expressing porcine APN via endocytosis and acidification of the intracellular compartment facilitated membrane fusion (Hansen et al., 1998). FIPV also requires acidification of endosomes for successful entry (Takano et al., 2008). Inhibition of FIPV infection with nystatin, a pharmacological reagent that causes caveolae to flatten and disrupt the coat structure, and dynamin 2 inhibitor suggests that FIPV entry might actually involve some types of caveolae-dependent endocytosis (Van Hamme et al., 2008).

Although several studies have examined the mechanism of entry of other coronaviruses, the mechanism of PEDV entry is still unknown. In this study, we studied the entry mechanism of PEDV by measuring virus infectivity in the presence of chemical inhibitors and co-localization of PEDV with endocytic pathway markers. We found that PEDV infection was diminished by treatment with chloropromazine (CPZ) and lysosomotropic agents. In addition, we also investigated that PEDV required serine-like proteases for their entry through endocytosis and for cell–cell fusion. Taken together, our findings reveal that PEDV enters Vero cells via clathrin-mediated endocytosis and requires serine proteolysis during infection.

2. Materials and methods

2.1. Cells and viruses

Vero cells were maintained in Eagle's minimum essential medium (MEM, Gibco) containing with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 34 μg/ml amphotericin B. KPEDV-9, a Vero cell-adapted Korean strain, was propagated in Vero cells as described previously (Hofmann and Wyler, 1988). Briefly, Vero cells were inoculated with the KPEDV-9 at a multiplicity of infection (MOI) of 10 and cultured in serum-free MEM for 72 h at 37 °C with 5% CO_2. The progeny viruses were titrated using the focus formation assay following a method described previously (Cruz and Shin, 2007).

2.2. Effect of trypsin in PEDV infection

KPEDV-9 infection in Vero cells under trypsin and non-trypsin conditions was compared for 48 h. Vero cells in 4-well tissue culture (TC) plate (SPL Labware) were inoculated with KPEDV-9 and cultured in either serum-free MEM or MEM supplemented with trypsin (10 μg/ml). Infection was stopped by addition of 5% paraformaldehyde (PFA) at the indicated times for immunocytochemistry.

2.3. Treatment with endocytosis inhibitors

Vero cells were treated with various concentrations of either CPZ for 30 min or 0.45 M sucrose for 10 min to inhibit the formation of clathrin-coated pits. To block the caveolae-dependent pathway, cells were incubated with various concentrations of nystatin for 30 min. Control cells were incubated with or without dimethyl sulfoxide (DMSO). Cells were inoculated with KPEDV-9 at a MOI of 10 for 2 h, and then overlaid with 0.5% methyl cellulose in MEM containing trypsin for 10 h. At 10 hpi, PEDV-infected cells were detected by immunocytochemistry.

2.4. Co-localization of PEDV with endocytic markers

To prepare ultra-purified trypsin-free viruses, Vero cells were inoculated with the KPEDV-9 at a MOI of 10 and cultured in serum-free MEM for 72 h. Supernatant was clarified by centrifugation at 20,000 × *g* for 20 min at 4 °C, followed by ultra-centrifugation using a 20% sucrose cushion at 150,000 × *g* for 3.5 h. Following resuspension in buffer A (1 M Tris, pH 8, 5 M NaCl, 0.1 M $CaCl_2$), protein concentration of purified virus stock was determined by the Bradford assay. Fluorochrome conjugation of KPEDV-9 with Alexa Fluor 594 (AF594) carboxylic acid-succinimidyl ester (Molecular probes) was performed according to manufacturer's instructions. Briefly, 5.0 mg of ultrapurified KPEDV-9 was dialyzed in labeling buffer (0.1 M $NaHCO_3$, pH 8.3) at 4 °C overnight. Virus was then incubated for 1 h on a platform rocker at room temperature with 1 μg of AF594 succinimidyl ester in 100 μl of DMSO. The AF594-labeled KPEDV-9 was extensively dialyzed in buffer A.

Vero cells were prepared on cover glasses a day before assay. For AF594-KPEDV-9 co-localization with endocytic markers, the cells were incubated with AF594-KPEDV-9 combination with 10 μg/ml of Alexa Fluor 488-conjugated transferrin (AF488-Tf) or 5.0 μg/ml of Alexa Fluor 488-cholera toxin subunit B (AF488-CT-B) for 30 min on ice to synchronize entry, and then shifted to 37 °C. Unbound viruses were removed, and the cells were fixed in 2% PFA at indicated times and analyzed at magnification of 63× on the laser scanning confocal microscope.

2.5. Neutralization of intracellular pH

Vero cells were treated with either 50 mM NH_4Cl or 1 μg/ml Baf-A1 to neutralize the intracellular pH. The cells were then inoculated with KPEDV-9 at a MOI of 10 for 2 h in the presence of lysosomotropic agents. The virus inoculums were removed by washing with PBS. Cells were then overlaid with 0.5% methyl cellulose in MEM containing trypsin for 10 h. At 10 hpi, PEDV-infected cells were detected by immunocytochemistry.

The effect of low pH on the fusion activity of the S protein was investigated by subjecting PEDV-infected Vero cells to a low pH range. Vero cells were inoculated with KPEDV-9 and cultured in trypsin-free MEM for 20 h. Afterwards, the cell monolayer was washed thrice with PBS and replenished with serum-free MEM adjusted to pH 3, 4, 5, 6, 7. MEM containing trypsin (10 μg/ml) at pH 7 was used as positive control. The cultures were further incubated at 37 °C for 4 h, and then fixed with 5% PFA. PEDV-infected cells were detected by immunocytochemistry.

2.6. Protease inhibitor assay

Cells were pretreated with various protease inhibitors such as E-64 (10 μM), AEBSF-HCl (500 μM), pepstatin A (10 μg/ml), and phosphoramidon (10 μM) for 1 h. For examination the synergistic antiviral activity of AEBSF-HCL and lysosomotropic agent, cells were treated with AEBSF-HCl and/or NH_4Cl for 1 h. Treated cells were then infected with KPEDV-9 at a MOI of 1 for 1 h in the presence of inhibitors. After 1 h adsorption, virus inoculums were removed by washing with PBS. Cells were then overlaid with 0.5% methyl cellulose in MEM containing trypsin for 10 h. At 10 hpi, PEDV-infected cells were detected by immunocytochemistry.