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Egg drop syndrome virus enters duck embryonic fibroblast cells via clathrin-mediated endocytosis

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ABSTRACT

Previous studies of egg drop syndrome virus (EDSV) is restricted to serological surveys, disease diagnostics, and complete viral genome analysis. Consequently, the infection characteristics and entry routes of EDSV are poorly understood. Therefore, we aimed to explore the entry pathway of EDSV into duck embryonic fibroblast (DEF) cells as well as the infection characteristics and proliferation of EDSV in primary DEF and primary chicken embryo liver (CEL) cells. Transmission electron microscopy revealed that the virus triggered DEF cell membrane invagination as early as 10 min post-infection and that integrated endocytic vesicles formed at 20 min post-infection. The virus yield in EDSV-infected DEF cells treated with chlorpromazine (CPZ), sucrose, methyl-β-cyclodextrin (MβCD), or NH_4Cl was measured by quantitative real-time PCR. Compared with the mock treatment, CPZ and sucrose greatly inhibited the production of viral progeny in a dose-dependent manner, while MβCD treatment did not result in a significant difference. Furthermore, NH_4Cl had a strong inhibitory effect on the production of EDSV progeny. In addition, indirect immunofluorescence demonstrated that virus particles clustered on the surface of DEF cells treated with CPZ or sucrose. These results indicate that EDSV enters DEF cells through clathrin-mediated endocytosis followed by a pH-dependent step, which is similar to the mechanism of entry of human adenovirus types 2 and 5.

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1. Introduction

Endocytosis is a crucial cellular function by which cells internalize extracellular “cargo” such as particles, fluid, and ligands (Conner and Schmid, 2003). Recent research has provided new insights into this process. In particular, it has been shown that many pathogens can utilize endocytosis as an entry point into target cells (Marsh and Pelchen-Matthews, 2000). The process of virus entry via endocytosis is complex, involving hundreds of cellular proteins. Many viruses gain entry into target cells by triggering the initiation of a number of endocytic pathways, including clathrin-mediated endocytosis (Spoden et al., 2008), caveolae/lipid raft-mediated endocytosis (Beer et al., 2005), macropinocytosis (Nanbo et al., 2010; Rossman et al., 2012), and several other unusual pathways. Clathrin-mediated endocytosis is the best characterized pathway (Doherty and McMahon, 2009). In this mode of vesicular transport, the viruses to be internalized bind to specific cell-surface receptors, and then the interaction between virus and receptor induces the nucleation of clathrin-coated pits, which leads to the formation of clathrin-coated vesicles (Ramanan et al., 2011). Several viruses, such as human immunodeficiency virus (Miyauchi et al., 2009), influenza virus (Chen and Zhuang, 2008), hepatitis C virus (Blanchard et al., 2006), bovine ephemeral fever virus (Cheng et al., 2012), rabies virus (Piccinotti et al., 2013), and human adenovirus types 2 and 5 (Ad2 and Ad5) can enter target cells through clathrin-mediated endocytosis (Patterson and Russell, 1983).

Egg drop syndrome virus (EDSV) is a member of the genus *Atadenovirus* within the family *Adenoviridae*. It is composed of double-stranded DNA and a non-enveloped icosahedral nucleocapsid of approximately 70–80 nm in diameter. It was first reported in chickens in the 1970s (Van Eck et al., 1976). EDSV primarily causes a sudden severe drop in egg production as well as the production of shell-less, thin-shelled, discolored, or misshapen eggs in apparently healthy laying birds (Hafez, 2011). Egg drop syndrome still occurs sporadically among birds. In 2001, EDSV infected young goslings between 4 and 20 days old and caused severe acute respiratory symptoms including anorexia, depression, coughing, sneezing, dyspnea, and rales (Ivanics et al., 2001). In 2007, an outbreak of egg

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drop syndrome occurred naturally in turkey breeder flocks and was accompanied by a significant decrease in both egg production and quality (Biđin et al., 2007). Recently, Cha et al. (2013) reported that EDSV infected 10-day-old Pekin ducklings and caused severe acute respiratory symptoms.

At present, the entry pathway of EDSV into target cells remains unclear. The aim of this study was to investigate the entry route of EDSV into duck embryonic fibroblast (DEF) cells. For this work, we utilized transmission electron microscopy (TEM) to observe the structural changes in DEF cells triggered by EDSV infection. Additionally, the cytopathic effect (CPE) of EDSV on infected DEF and primary chicken embryo liver (CEL) cells was examined by optical microscopy. Quantitative PCR was used to detect changes in virus titers when cells were treated with inhibitors that interfere with endocytosis or endosome acidification. Furthermore, we used indirect immunofluorescence to observe changes in virus distribution on cells treated with chlorpromazine or sucrose as compared with control cells. The results indicate that EDSV enters cells via clathrin-mediated endocytosis followed by a pH-dependent step.

2. Materials and methods

2.1. Virus and cells

EDSV strain 127 was propagated in the allantoic fluid of 10-day-old specific-pathogen-free embryonated duck eggs at 38.5 °C for 120 h (Green Square Biological Engineering Company, China). The hemagglutination titer of the allantoic fluid was tested using 1% chicken erythrocytes, and the allantoic fluid was stored at −80 °C.

Primary DEF cells were obtained from 12- to 14-day-old duck embryos according to a previously described method (Docherty and Slota, 1998). Primary CEL cells were obtained from 10-day-old chick embryos using previously described methods (Lincoln et al., 1988) with slight modifications. DEF cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and CEL cells were grown in Waymouth's medium with 10% fetal bovine serum. For experiments, 1.0×10^6 cells were seeded in 6 cm culture dishes in a 37 °C incubator with 5% CO_2. All experimental procedures were started when cell confluence reached approximately 80%. DEF and CEL cells were infected with EDSV at a multiplicity of infection (MOI) of 2. The CPE was observed with an optical microscope. In addition, supernatant from DEF and CEL cells infected with EDSV was collected every 12 h and stored at −80 °C for analysis of EDSV proliferation.

2.2. Antibodies

Mouse anti-EDSV hyperimmune serum was prepared according to a slightly modified procedure for mouse immunization (Zhang et al., 2011). Briefly, the allantoic fluid containing EDSV was concentrated and purified using the two-step cesium chloride density ultracentrifugation method (Luo et al., 2007). The titer of purified EDSV particles was determined by measuring the absorbance at 260 nm, in accordance with a previous study (Tian et al., 2007). Female Kunming white mice (Fourth Military Medical University, China), 4–6 weeks old, were immunized subcutaneously with 1.0×10^8 EDSV particles emulsified with complete Freund's adjuvant (Sigma-Aldrich Co., USA). The second immunization was given 15 days later, using the same amount of antigen emulsified with incomplete Freund's adjuvant (Sigma–Aldrich Co.). This was followed by two booster doses of antigen emulsified with incomplete Freund's adjuvant at 10-day intervals. Ten days after the last immunization, the hyperimmune serum was aseptically collected and the antibody titer was measured by hemagglutination inhibition assay. The mouse anti-EDSV hyperimmune serum was stored at −80 °C. Fluorescent antibody (goat anti-mouse IgG conjugated to iFluor 555 [IF555]) was purchased from Sungene Biotech Co. (China).

2.3. Chemical inhibitors

Inhibitors of endocytosis and endosome acidification were used to study the route of EDSV entry into DEF cells. Chlorpromazine (CPZ; an inhibitor of clathrin-mediated endocytosis) (catalog number 31,679), methyl-β-cyclodextrin (MβCD; a reagent that disrupts both caveolin-dependent endocytosis and caveolin-independent lipid raft-dependent endocytosis) (catalog number C4555), NH_4Cl (a drug that interferes with endosome acidification), and sucrose were purchased from Sigma–Aldrich Co.

2.4. Establishment of a standard curve for quantitative real-time PCR to detect EDSV

Viral DNA was extracted from the allantoic fluid of EDSV-inoculated duck embryos using the TIANamp Virus DNA/RNA Kit according to the manufacturer's instructions (TIANGEN Biotech Co., China). Primers based on the highly conserved penton gene of strain 127 (GenBank accession No. Y09598.1) were designed using Primer Premier 5.0 software. The forward and reverse primer sequences were 5′-CGTTCGCCTAATGACT-3′ (identical to nucleotides 12100–12115) and 5′-CTGCCTTCCAACTTTC-3′ (complementary to nucleotides 12250–12235), respectively. The length of the amplified fragment was 151 bp. The PCR conditions were as follows: hold for 10 min at 94 °C, followed by 35 cycles of 15 s at 94 °C, 30 s at 50 °C, and 12 s at 72 °C. The PCR products were cloned into the pMD-19T cloning vector (Takara Biotech Corporation, China) and recombinant vectors were transformed into competent *E. coli* DH5α cells (Takara Biotech Corporation). The recombinant plasmid DNA was extracted using the E.Z.N.A.® Plasmid DNA Mini Kit I (Omega Bio-tek, USA) and sequenced by Invitrogen (Invitrogen Biotechnology Co., China). The concentration and purity of recombinant plasmid DNA were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). The copy number of the recombinant plasmid was calculated as described previously (Ke et al., 2006). The standard plasmid DNA was diluted in a 10-fold dilution series ranging from 1.0×10^2 to 1.0×10^8 copies/μL and measured in triplicate using TransStart Top Green qPCR SuperMix (Transgen Biotech Co., China). The standard curves were generated with an iCycler iQ5 real-time PCR detection system (Bio-Rad, USA). The correlation coefficient (R) and PCR efficiency were also determined.

2.5. Electron microscopy of EDSV particle uptake into host cells

Ultra-thin sections (70 nm) of cells were prepared and examined by TEM as described previously (Ishii et al., 2010). Briefly, DEF cells (1×10^6) in 6 cm culture plates were inoculated with EDSV (MOI of 5000 particles/cell) and incubated at 4 °C for 2 h. One milliliter of DMEM with 2% fetal bovine serum was added to the cells and then the cells were incubated at 37 °C for 0 min, 10 min, 20 min, 30 min, 2 h, and 72 h. After incubation, the cells were collected by centrifugation at $800 \times g$ for 5 min, and one drop of 2% preheated agarose was uniformly mixed with the cell pellet. After solidification, 1 mm^3 agarose blocks were cut, fixed in 2.5% glutaraldehyde/0.1 M phosphate buffer solution at 4 °C, washed three times with phosphate buffer solution for 10 min, and post-fixed in 1% osmium tetroxide at 4 °C for 1 h. Following dehydration in a graded series of ethanol solutions, the cells were embedded in a mixture of epoxy 812 and warmed at 35 °C, 45 °C, and 60 °C for 12 h, 12 h, and 48 h, respectively. Ultra-thin sections were prepared and stained with 4% uranyl acetate for observation under a Hitachi HT-

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