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Epidermal growth factor receptor is a co-factor for transmissible gastroenteritis virus entry

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ABSTRACT

Transmissible gastroenteritis virus (TGEV) causes severe diarrhea and high mortality in newborn piglets. It is well established that porcine intestinal epithelium is the target of the TGEV infection, however the mechanism that TGEV invades the host epithelium remains largely unknown. Aminopeptidase N (APN) is a known receptor of TGEV. This study discovered that the extracellular receptor binding domain 1 pertaining to epidermal growth receptor (EGFR) interact with TGEV spike protein. APN and EGFR synergistically promote TGEV invasion. TGEV promotes APN and EGFR clustering early in infection. Furthermore APN and EGFR synergistically stimulate PI3K/AKT as well as MEK/ERK1/2 endocytosis signaling pathways. TGEV entry is via clathrin and caveolin mediated endocytosis in IPEC-J2 cells. TGEV binds with EGFR, and subsequently promotes EGFR internalization by a clathrin-mediated endocytosis pathway. These results show that EGFR is a co-factor of TGEV, and that it plays a synergistic role with APN early in TGEV infection.

1. Introduction

Procine transmissible gastroenteritis virus (TGEV) is a member of the enteropathogenic alpha-coronavirus (αCoV) family. TGEV infects intestinal epithelial cells resulting in severe and frequently fatal diarrhea in newborn pigs, with mortality rates reaching 100% (Doyle and Hutchings, 1946). TGEV is an enveloped CoV, with a large positive-sense single-stranded RNA genome, about 28.5 kb in length. It has a diameter ranging from 80 to 120 nm, including surface projections. Porcine intestinal columnar epithelial cells (IPEC-J2) offer a practical model for studying porcine enteric pathogens (Brosnahan and Brown, 2012). We will use this model to study the entrance mechanism of TGEV.

Aminopeptidase N (APN), also known as CD13, is a typeII-transmembrane glycoprotein, about 150 kDa, belonging to a membrane-bound metalloprotease family (Delmas et al., 1994). Most alpha coronavirus use APN as cellular receptors for virus entry, such as human coronavirus 229E (HCoV-229E), feline infectious peritonitis virus (FIPV), canine coronavirus (CCoV), porcine epidemic diarrhea virus (PEDV), and transmissible gastroenteritis virus (TGEV) (Delmas et al., 1992, 1993; Kolb et al., 1996; Li et al., 2007; Tresnan et al., 1996).

The high degree of tropism of TGEV for the villous enterocytes of newborn pigs is well established and has been suggested as being a factor in age sensitivity of newborn pigs to the virus (Schwegmann-Wessels and Herrler, 2006). There has been some confusion around the question that if APN is the only receptor for TGEV entry, why older piglets are not susceptible to TGEV, especially since APN was found to be highly expressed in villous enterocytes of both newborn and older piglets. Research suggest a known protein, approximately 200-kDa in size, only expressed in the upper villi of newborn piglets, it has high affinity for TGEV (Weingartl and Derbyshire, 1994). It has also been demonstrated that APN is not essential for PEDV cell entry (Ji et al., 2018; Li et al., 2017). It is most likely that TGEV do have more than one receptor.

Many cell surface components have been identified as virus receptors, including: chemokine receptors (Feng et al., 1996), fibroblast growth factor receptors (Qing et al., 1999), the tumor necrosis factor receptor family (Terry-Allison et al., 1998), and integrin (Wang et al., 2005). pidermal growth factor receptor (EGFR), a member of the receptor tyrosine kinases (RTK) family, is widely expressed on many cells including epithelial and mesenchymal cells (Wells, 1999b). It has been demonstrated that many viruses interact with EGFR to facilitate viral entrance, including: influenza A virus (IAV), hepatitis C virus (HCV), herpes simplex virus 1 (HSV), and human cytomegalovirus (HCMV) (Chan et al., 2009; Eierhoff et al., 2010a; Lupberger et al., 2011; Zheng

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et al., 2014b). Ligand binding to EGFR induce receptor dimerization and cross phosphorylation, which in turn actives the intracellular signaling cascades critical for cellular protein synthesis, cytoskeleton reorganisation, apoptotic inhibition, transcriptional activation, and cell motility. The ligand binding to EGFR results in rearrangement of the cytoskeleton network through EGFR-mediated signaling, and subsequently ligand-EGFR complexes are internalize through clathrin-coated pits (Zheng et al., 2014a). Many researchers have shown that numerous viruses utilize EGFR endocytosis to mediate virus internalization (Mercer et al., 2010a). The interactions between viruses and their receptors are specific, but the affinity is low. Many multiple receptor binding sites exist on virus particles which are likely to cluster receptor proteins. It is known that multiple viruses use more than one type receptor to aid uptake into host cells (Marsh and Helenius, 2006). EGFR also has been identified as a co-receptor for many viruses, such as human cytomegalovirus (HCMV), hepatitis C virus (HCV), and adeno-associated virus serotype 6 (AAV6) (Lupberger et al., 2011; Wang et al., 2003; Weller et al., 2010). Previous studies that we have conducted demonstrated that TGEV spike protein interacts with EGFR, and stimulates phosphorylation of cofilin as well as stimulating polymerization of F-actin through the PI3K-Rac1/Cdc42-PAK-LIMK signaling pathway. This is required for efficient TGEV entry (Hu et al., 2016). We further demonstrate whether EGFR is another co-factor for TGEV. EGFR is a transmembrane protein with two dimer forms, it can be divided into extracellular, transmembranal, and intracellular regions. Its extracellular region contains two receptor-binding domains Receptor 1 (57-168 aa) and Receptor 2 (361-481 aa). Its intracellular protein associated with tyrosine kinase PI3K/Akt and Ras/Raf/ERK1/2 pathways are activated by phosphorylated tyrosine located in EGFR cytoplasmic tails (Fig. 1A). The objective of our present study was also to study the relationship between APN and EGFR in the early stage of TGEV infection.

2. Materials and methods

2.1. Cells culture

IPEC-J2 cells are porcine intestinal columnar epithelial cells that are isolated from the middle jejunum of neonatal piglets. IPEC-J2 cells were purchased from DSMZ (Germany). HEK293T cells and swine testis (ST) cells were purchased from ATCC (United States). IPEC-J2, ST, and HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, HEPES containing with 10% fetal bovine serum (FBS, GIBCO), 1% penicillin-streptomycin (Invitrogen) at 37 °C in a 5% CO_2 incubator (ThermoFisher Scientific).

2.2. Virus infection and assays

Transmissible gastroenteritis virus (strain SHXB) was isolated in Shanghai, China. The complete genome sequence for TGEV SHXB is available in GenBank (KP202848.1) (Weiwei et al., 2014). To analyze viral entry, cells were incubated with TGEV at a multiplicity of infection of 2 (MOI = 2) for 1 h at 4 °C. Subsequently, the cells were washed with phosphate-buffered saline (PBS), and maintained in a maintenance medium (DMEM supplemented with 2% FBS and 1% penicillin-streptomycin) for 1 h at 37 °C in a 5% CO_2 incubator.

For viral labeling, viruses were filtered with 0.22 μm filter, and then clarified by centrifugation at 10,000*g* for 2.5 h, followed by ultra-centrifugation using 20%, 40%, and 60% sucrose gradient at 10,000*g* for 2.5 h. Viruses were labeled with the fluorescent probe DyLight 488, 633, and 594 NHS Ester (ThermoFisher Scientific, Waltham, USA), according to the manufacturer's instruction. Unincorporated dye was removed by using commercial fluorescent dye removal columns (ThermoFisher Scientific).

Flow cytometry analysis for the entry of TGEV was performed as follows: Fluorescent probe labeled “TGEV particles” were incubated with IPEC-J2 cells for 1 h at 4 °C. Subsequently, the cells were washed with PBS and maintained in DMEM for 1 h at 37 °C in a 5% CO_2 incubator, the cells were harvested by 0.25% trypsin, and then washed with PBS three times. Cells acquisition was performed by FACS (Becton Dickinson), and the date was analyzed using Flowjo software.

2.3. Antibodies and western blotting

Antibodies used in the present study were obtained from commercial sources. These antibodies included: rabbit anti-human EGFR, rabbit anti-human phospho-EGFR (Tyr1068), rabbit anti-human AKT, rabbit anti-human phospho-AKT, rabbit anti-human phospho-ERK1/2, and rabbit anti-human ERK1/2 (Cell Signaling Technology, Danvers, USA). Mouse anti-procine APN antibody was donated by prof. Zhu Guoqiang in Yangzhou University. Mouse monoclonal antibodies to HA and GFP (CMCTAG, Milwaukee, USA). Goat anti-rabbit IgG (H + L) secondary Antibody, DyLight 594 conjugate, goat anti-mouse IgG (H + L) secondary Antibody, DyLight 488 conjugate (ThermoFisher Scientific). Anti-GAPDH monoclonal antibody, HRP-conjugated goat anti-rabbit IgG (H + L), and HRP-conjugated Goat Anti-Mouse IgG (H + L) (Vazyme, Nanjing, China).

IPEC-J2 cells were washed with PBS and lysed in an ice-cold cell lysis buffer, phosphatase inhibitor and protease inhibitor (ThermoFisher Scientific) were added in the cell lysis buffer according to the manufacturer's instructions. The supernatant of lysates were obtained by centrifugation at 12,000*g* for 10 min at 4 °C, and subsequently equal protein levels of the prepared lysates were fractionated by SDS-PAGE (10–12% gradient). The separated proteins were transferred to PVDF (Merck Millipore), and the membranes were blocked for 2 h in Tris-buffered saline (TBS), containing 5% nonfat dry milk. After which they were reacted with indicated primary antibodies at 4 °C overnight. Membranes were exposed to species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies, (dilution 1:5000) followed by enhanced chemiluminescence (ECL, Thermofisher Scientific) detection by use of autoradiography. Western blotting was quantified by Quantity One (Quantity One 1-D Analysis Software 170-9600, Bio-Rad). The intensity of the bands in terms of density was measured and normalized against GAPDH expression. All data were expressed as means ± SD of three independent experiments.

2.4. Plasmid construction and DNA transfection

The pLVX-DsRed-Monomer-N1 is an HIV-1-based, lentiviral expression vector that expresses the gene of interest fused to the DsRed-Monomer (Clontech, Palo Aito, CA). EGFR and APN sequences were inserted into the EcoRI/BamHI site. EGFR receptor1 and EGFR receptor2 sequences were cloned into a pAcGFP1-C vector (SalI/BamHI) (Clontech), and TGEV Spike1 sequence was cloned into pCMV-C-HA (BamHI/XbaI) (D2639, Beyotime, China). Table 2 showed the primers used for cloning. All constructs were verified by DNA sequencing. HEK 293 T cells were optimized for lentivirus production, we transfected APN or EGFR lentiviral overexpression vector and Lenti-X HTX Packaging Mix (VSV-G, plp1, plp2) into HEK 293T cells using the X-tremeGENE HP DNA Transfection Reagent (Roche, Switzerland), according to the manufacturer's instructions. IPEC-J2 cells were treated with APN, EGFR overexpressing lentiviral particles (MOI = 1), and after 24 h of incubation, infected cells were maintained with fresh DMEM, and continued for extra 12–24 h to allow the overexpressing lentiviral particles to achieve their maximum effect.

2.5. His-EGFR Receptor1 and His-EGFR Receptor2 expression and purification

His-EGFR Receptor1 and His-EGFR Receptor2 were cloned into and expressed in *Escherichia coli* BL-21, then purified using Ni-NTA resin. The purified proteins were eluted with elution buffer containing 8 M