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Efficient porcine reproductive and respiratory syndrome virus entry in MARC-145 cells requires EGFR-PI3K-AKT-LIMK1-COFILIN signaling pathway

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ABSTRACT

Viruses have evolved diverse strategies to take over cellular machinery to facilitate their infection. In our studies presented here, we first demonstrated that Src kinase was involved in PRRSV entry in MARC-145 cells. Further studies demonstrated epidermal growth factor receptor (EGFR) was activated by the currently unknown mechanism(s) during PRRSV entry, which subsequently initiated EGFR downstream signal pathways, such as PI3K/AKT/LIMK1. Through these pathways, the virus entry signal was ultimately transferred to cofilin, which might regulate the actin fragmentation and reorganization to facilitate the virus penetration and cytoplasmic trafficking.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative pathogen for porcine reproductive and respiratory syndrome (PRRS), an infectious disease that causes major economic impact in the swine industry worldwide (Neumann et al., 2005). PRRSV is a positive-sense RNA virus that belongs to the family of *Arteriviridae* and the order of *Nidovirales* (Cavanagh, 1997; Conzelmann et al., 1993; Meulenberg et al., 1993). The viral genome is about 15 kb in length, which consists of the 5′ un-translated region (UTR), ten open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, and ORFs 3 through 7, including ORF5a), and the 3′ UTR (Johnson et al., 2011). The ORFs 1a and 1b account for 75% of the viral genome and encode two long polypeptides (pp), pp1a and pp1ab; after enzymatic cleavage, the polypeptides produce 14 non-structural proteins (nsps) that are responsible for viral replication. The ORFs 2–7 encode eight structural proteins, including GP2a, GP2b, GP3, GP4, GP5, GP5a, matrix protein (M) and nucleocapsid protein (N) (Allende et al., 1999; Firth et al., 2011; Johnson et al., 2011), which are mainly associated with virus entry, assembling, and release.

Viruses have evolved diverse strategies to take over cellular machinery to facilitate their infection. It has been demonstrated that PRRSV infects the target cells through receptor-mediated endocytosis (Nauwynck et al., 1999). Heparin sulfate, sialoadhesin (Sn), CD163, CD151 and vimentin have been identified as PRRSV receptors, and they play different roles in PRRSV infection. Heparan sulfate glycosaminoglycans (GAGs) on the macrophage surface are assumed to function as PRRSV attachment factors that concentrate virions on the cell surface, hence allowing a more efficient infection. Sialic acids on the viral envelope proteins interact with the porcine Sn receptor on the macrophage surface, thereby triggering internalization of the virus. Upon binding to sialoadhesin, the virus is internalized via clathrin-mediated endocytosis (Delputte and Nauwynck, 2004; Nauwynck et al., 1999). CD163 is responsible for uncoating virus particles and releasing the viral genome (Van Gorp et al., 2008, 2010; Welch and Calvert, 2010). An acidic pH is required to trigger a fusion event between the viral envelope and the endosomal membrane to facilitate viral genome release (Kreutz and Ackermann, 1996; Nauwynck et al., 1999). Further identifica-

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tion of additional molecules involved in virus entry may contribute to the understanding of virus infection process.

The host-virus interaction leads to the activation of various cellular signaling pathways. Src-family kinases regulate numerous cellular processes, such as proliferation, differentiation, migration, adhesion, and cytoskeletal rearrangements (Thomas and Brugge, 1997). Src kinases also mediate cell entry of both enveloped and non-enveloped viruses, including human immunodeficiency virus (HIV) (Tokunaga et al., 1998), Kaposi's sarcoma-associated herpesvirus (Veettil et al., 2006), coxsackievirus (Coyne and Bergelson, 2006) and avian reovirus (Ping-Yuan et al., 2006). Src is the prototype member of this family, which was first identified as the oncoprotein of Rous sarcoma virus (Brugge and Erikson, 1977; Purchio et al., 1978). It has been revealed that Src interacts with a number of receptor tyrosine kinases, including epidermal growth factor receptor (EGFR) (Luttrell et al., 1988). EGFR is a member of ERBB/HER receptor family with intrinsic tyrosine kinase activity. Tyrosine autophosphorylation within the cytoplasmic tail of EGFR can be used as the binding sites for proteins containing Src homology 2 and phosphotyrosine-binding domains (O'Bryan et al., 1998). Activation of EGFR increases Src catalytic activity (Weernink and Rijksen, 1995). Src, in turn, phosphorylates EGFR to activate its downstream signal pathways (Biscardi et al., 1999; Sato et al., 1995b; Stover et al., 1995). Specifically, phosphorylation of EGFR by Src promotes phosphoinositide-3 kinase (PI3K) binding to EGFR, leading to Akt phosphorylation and cellular survival signaling (Stover et al., 1995). The PI3K/Akt pathway is critical in regulating for Ebola virus entry (Saeed et al., 2008).

Cofilin (CFL) is a member of the actin depolymerizing factor family, which binds to both monomeric globular actin and filamentous actin and regulates severance and depolymerization of actin filaments (Bernstein and Bamburg, 2010; Moon and Drubin, 1995; Van Troys et al., 2008). Phosphorylation at Ser-3 by LIM kinase 1 inactivates cofilin, while dephosphorylation at Ser-3 by phosphatase Slingshot (SSH) reactivates cofilin. Regulation of cofilin function through phosphorylation and dephosphorylation transmits extracellular signals to the actin cytoskeleton. Meanwhile, phosphatidylinositol 3-kinase (PI3K) can also regulate cytoskeletal reorganization. PI3K-Akt signaling pathway has been shown to regulate early cofilin phosphorylation during herpes simplex virus 1 entry into neuronal cells (Zheng et al., 2014).

The entry is an important part of virus life cycle which can be targeted for future antiviral development. However, precise mechanisms of how cell signaling pathways regulate PRRSV entry remain unclear. In our studies presented here, we first examined the involvement of Src kinase in PRRSV entry in MARC-145 cells. Next, we made an attempt to dissect the mechanisms of how Src kinase affects PRRSV entry. Our investigation suggested that EGFR, PI3K/AKT, and LIMK1 signaling pathway participated in the PRRSV entry, which regulated activity of cofilin to facilitate virus entry.

2. Materials and methods

2.1. Cell, virus, and reagents

MARC-145 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Porcine reproductive and respiratory syndrome virus (JXA1 strain) was propagated in MARC-145 cells and stored at −80 °C until use. The N-specific monoclonal antibody was kindly provided by Prof. Jia-qiang Wu. Virus infectivity was assessed in MARC-145 cells using plaque formation assay. The tyrosine kinase inhibitor genistein was purchased from Sigma. PP2, SU6656, AG1478, LY294002, PI3K activator, and Y27632 were purchased from Santa Cruz Biotechnology. The inhibitors were dissolved either in water or DMSO and used at the indicated concentration. Protease inhibitor tablet was obtained from Roche. The antibodies for phospho-Src (Y416), Src, phospho-EGFR (Y1068), EGFR, phospho-Akt (S473), Akt, phospho-cofilin (Ser3) and cofilin were obtained from Cell Signaling Technology. The phospho-LIMK1 (Thr 508) and LIMK1 antibodies were obtained from Santa Cruz Biotechnology. The antibodies targeting HA, GFP and β-actin were purchased from Sigma. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Thermo Scientific. Anti-EGFR neutralizing antibody (clone LA1) was purchased from Millipore. The human EGF was purchased from Invitrogen. HRP-rec-Protein G was purchased from Thermo Fisher Scientific (Lal et al., 2005). Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen) was used as secondary antibody.

Table 1
The primer sequences used for PCR, qRT-PCR or siRNA sequence.

Name	Primer sequence (5′ to 3′)
Cofilin S3A	Forward: GTACATCGAATTCATGGCCGCCGGTGTGGCTGTCTCCGA CGGCGTGATCAAGGTGTTCA Reverse: GATGCATCTCGAGTCACAAAGGCTTGCCCTCCAGGGAGATGAC
siCFL	CTGTCTCTGATGGTGTCAT
Viral N gene	Forward: TTGTGTCTGTCGTCGATCCAG Reverse: AAACTCCACAGTGTAACTTATCCTC Probe: (FAM) CGCTGGAACTTGTGCCCTGTCA (Eclipse)
β-Actin	Forward: TGACTGACTACCTCATGAAGATCC Reverse: TCTCCTTAATGTCACGCACGATT Probe:(FAM)CGGCTACAGCTTCACCACCACGGC Eclipse)

2.2. Plasmid constructs, siRNA, and transfection

Constitutively active cofilin (S3A, siRNA-resistant) was cloned into pCAGGS-HA vector through *EcoR*I and *Xho*I restriction sites (Table 1). The construct fidelity was confirmed by DNA sequencing. GFP-EGFR was kindly provided by Prof. Rockman (Duke University). SiRNA targeting Cofilin (siCFL) was purchased from Biotend (Shanghai, China) (Table 1). MARC-145 cells were transfected with TransIT®-2020 Transfection Reagent (Mirus Bio) according to the manufacturer's instructions. Briefly, siRNA or plasmid DNA solution was mixed with TransIT-2020 in Opti-MEM I Reduced-Serum Medium. The mixture was added drop-wise to cells (5×10^5 cells/6-well plate, 60–80% confluency). The cells were incubated for 24–72 h before harvest and analysis.

2.3. Immunofluorescence assay (IFA)

MARC-145 cells grown on microscope coverslips were placed in 6-well tissue culture plates and pretreated with the Src inhibitors, genistein (50 μM), PP2 (10 μM) and SU6656 (5 μM) for 1 h before infected with PRRSV (MOI = 0.1). The virus infected cells were further grown in the presence of each inhibitor or DMSO for 24 h. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS at room temperature for 5 min. The cells were blocked using 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature and then incubated with N-specific MAb for 2 h. The cells were incubated with Alexa Fluor 488 conjugated goat anti-mouse secondary antibody for 1 h at room temperature after PBS wash. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Beyotime Biotechnology). The cells were observed under a fluorescence microscope (Carl Zeiss). Infected cells were shown by cytoplasmic fluorescence staining and quantified by scoring four random fields with equivalently confluent monolayers in triplicate wells.