



Syndecan-4, a PRRSV attachment factor, mediates PRRSV entry through its interaction with EGFR



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ARTICLE INFO

Article history:

Received 2 May 2016

Accepted 16 May 2016

Available online 18 May 2016

Keywords:

Porcine reproductive and respiratory syndrome virus
Attachment
Entry
Syndecan-4
EGFR

ABSTRACT

The causative agent of porcine reproductive and respiratory syndrome is the PRRS virus (PRRSV), an enveloped, single-stranded and positive-sense RNA virus. The host factors and mechanisms that are involved in PRRSV entry are still largely unknown. In our present studies, we found that syndecan-4, one of the heparan sulfate proteoglycans, plays a critical role in PRRSV entry, especially in PRRSV attachment. Moreover, EGFR interacts with syndecan-4 in MARC-145 cells and disruption of their interaction impaired PRRSV entry. Furthermore, EGFR inhibitor AG1478 or syndecan-4 derived peptide SSTN87-131 inhibited syndecan-4 endocytosis induced by PRRSV entry. Altogether, syndecan-4, a PRRSV attachment factor, mediated PRRSV entry by interacting with EGFR.

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS), which emerged in the late 1980s in Europe and North America, is a devastating swine disease which causes the significant economic impacts in the pig industry worldwide [1,2]. PRRS results in reproductive failure in pregnant sows and respiratory distress in unweaned and growing pigs [3,4]. The causative agent, PRRS virus (PRRSV), is an enveloped, single-stranded and positive-sense RNA virus, which belongs to the family of *Arteriviridae* in the order of *Nidovirales*. Other viruses in the same family include equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) [5,6]. PRRSV genome is approximately 15.4 kb, consisting of ten open reading frames (ORF1a, ORF1b, ORF2a, ORF2b and ORFs 3 through 7, including ORF5a) flanked by 5' and 3'-untranslated region [7]. Significant endeavors have been made to investigate the host immunological

responses during PRRSV infection, whereas the cellular factors and the mechanisms that are involved in the different stages of PRRSV infection are still largely unknown.

It has been demonstrated that PRRSV infects MARC-145 cells through receptor-mediated endocytosis [8]. To date, several important PRRSV receptors including heparin sulfate (HS), sialoadhesin (Sn), CD163, CD151 and vimentin have been suggested. These factors play the critical roles in the virus binding, internalization, or uncoating. Previous studies have shown that heparin, a highly sulfated form of HS, significantly reduced PRRSV infection in MARC-145 cells and PAMs [9–11]. Heparinase I (an enzyme that cleaves heparin and heparan sulfate) treatment prevents the virus infection in MARC-145 cells [9] or PAMs [12]. Moreover, HS has been proposed to function as the adhesion factor for PRRSV infection [11,12], whereas the molecule that carrying heparan sulfate has not been identified.

Syndecans are the largest group of heparan sulfate proteoglycans (HSPGs) on the surface endothelial cells [13], which are the only HSPG that penetrates the cytoplasm and interacts with the cytoskeleton [14]. It has been revealed that syndecan-1 and syndecan-4 capture epidermal growth factor receptor (EGFR) family members and integrin $\alpha\beta 1$ via binding sites in their ectodomains. EGFR is captured via the extracellular site comprised of amino acids 87–131 in human syndecan-4 [15].

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In our study, we explored the role of syndecan-4 (SDC4) in PRRSV attachment and entry in MARC-145 cells. We found knock-down of SDC4 significantly impaired PRRSV attachment, whereas SDC4 overexpression enhanced PRRSV attachment and entry. Our results showed that SDC4 interacted with EGFR in MARC-145 cells. Disruption of the interaction by the peptide derived from SDC4 (SSTN87-131) reduced PRRSV entry. In summary, we found that PRRSV initiated infection by attaching to SDC4, followed by virus entry with the help of the interaction between SDC4 and EGFR in MARC-145 cells.

2. Methods

2.1. Cells and virus

MARC-145 cells were grown in monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented to contain 10% fetal bovine serum (FBS, Gibco, USA), 100 U/ml of penicillin, 100 µg of streptomycin per ml. Porcine reproductive and respiratory syndrome virus PRRSV (JXA1 strain) was propagated in MARC-145 cells and stored at -80°C until use. Virus infectivity was assessed in MARC-145 cells using a plaque forming unit assay. Confluent monolayer cells were exposed to serial dilutions of virus for 1 h at 37°C . MARC-145 cells were overlaid with agarose and incubated for 72 h. The cells were stained with crystal violet and plaques were calculated.

2.2. Plasmid constructs, siRNA, and transfection

Syndecan-4 gene was amplified from cDNA that was synthesized by reverse transcription using total cellular RNA isolated from MARC-145 cells. The PCR product was digested with *EcoR*I and *Xho*I and ligated into pCAGGS-HA plasmid previously digested with the same restriction enzymes. The plasmid was designated as HA-SDC4. siRNAs targeting syndecan-4 (siSDC4) were purchased from Biotend (Shanghai, China). 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.

2.3. Chemical inhibitors, antibodies, and other reagents

EGFR inhibitor AG1478 and Syndecan-4 antibody were purchased from Santa Cruz Biotechnology. HA- and β -actin antibodies were obtained from Sigma. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Dingguo (China). Alexa Fluor[®] 488 goat anti-mouse IgG (H + L) (Invitrogen) was used as the secondary antibody. Syndecan-4 extracellular-domain peptide (SSTN87-131) was synthesized by GL Biochem Ltd (Shanghai, China).

2.4. PRRSV attachment and internalization assay

For attachment assay, MARC-145 cells were first incubated with virions at 4°C for 1 h. After extensive washing, the viral RNA was prepared using TRIzol (TaKaRa) according to the manufacturer's protocol. For virus entry assay, MARC-145 cells were first incubated with virions at 4°C for 1 h. The cells were washed and further incubated at 37°C with fresh medium. At indicated time points, cells were washed with citric acid buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) to remove the un-internalized virus particles. The cells were further washed with cold PBS. Total viral

RNA was extracted and measured by quantitative real-time PCR. The reactions were performed using the CFX96 Real-time PCR system (Bio-Rad). Each assay was run in triplicate. The data was analyzed using the relative expression ($2^{-\Delta\Delta\text{CT}}$ method) normalized to actin. The primer and probe sequences used are shown in Table 1.

2.5. Western blot analysis

MARC-145 cells grown in DMEM medium were transfected with pCAGGS-HA plasmid or HA-SDC4. At indicated time, monolayer cells were washed with PBS and lysed with lysis buffer (20 mM Tris-HCl, pH 8.0, 50 mM sodium chloride, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 20 mM sodium pyrophosphate, and 1 mM PMSF) containing protease inhibitors and phosphatase inhibitors cocktail (Thermo). Protein concentration was determined with the Bradford assay. Cell lysates were separated on SDS-PAGE gel and transferred to PVDF membrane. After blocking in 5% nonfat milk, the membrane was incubated with respective primary antibodies followed by HRP conjugated secondary antibodies. The immunoreactive bands were visualized by enhanced chemiluminescence system (Thermo).

2.6. Co-immunoprecipitation assay

Co-immunoprecipitation was carried out as previously described [16]. Briefly, cells (8×10^6) were washed twice with washing buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM EDTA) and then lysed in buffer (20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1% Triton X-100 and 0.5% Sodium deoxycholate, protease inhibitors and phosphatase inhibitors) for 20 min on ice. Cell debris was removed by centrifugation at 12,000 rpm for 15 min at 4°C . The supernatant was pre-cleared with 100 µl rProtein G Agarose (Invitrogen). Pre-cleared lysates were then incubated at 4°C overnight with either 10 µg/ml mouse monoclonal Syndecan-4 antibody or mouse IgG as negative control. The immune-complexes were incubated with rProtein G Agarose for 2 h, washed four times with washing buffer, resuspended in 60 µl of $2 \times$ SDS-PAGE sample buffer and boiled. The samples were subjected to SDS-PAGE and transferred to PVDF membrane. Anti-EGFR or anti-Syndecan-4 monoclonal antibodies were used to detect EGFR or Syndecan 4.

2.7. Immunofluorescence staining and confocal microscopy

Cells were challenged with virus at 4°C for 1 h and then

Table 1
The primer sequences used for quantitative real-time PCR.

Name	Primer sequence (5' to 3')
Syndecan-1	Forward: AAGATCAAGATGGCTCTG Reverse: GCTGTGACAAGGTGATAT
Syndecan-2	Forward: CATGCTGGTGGAGTTAT Reverse: GCTTCCTTCATCTTCTT
Syndecan-3	Forward: AGAAGAAGAGACCACACAAC Reverse: GTGGAGATGATGGCTTGG
Syndecan-4	Forward: CACTGAAACCAAGAAACTG Reverse: GTTAGACACATCCTCACTT
β -actin	Forward: TGAAGTACCTCATGAAGATCC Reverse: TCTCCTTAATGTACGCACGATT Probe:(FAM)CGGCTACAGCTTCACCACCACGGC (Eclipse)
PRRSV-N	Forward: TTGTGCTCTGTCGATCCAG Reverse: AAATCCACAGTGAATCTATCTC Probe:(FAM)CGTGGAAGTGTGCCTGTCA (Eclipse)

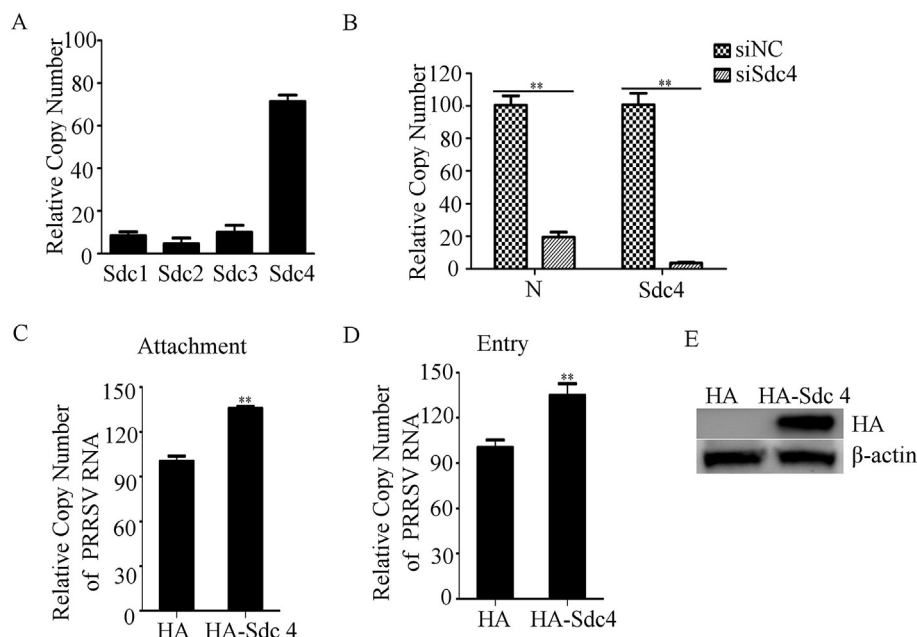


Fig. 1. (A) The mRNA levels of syndecan-1, 2, 3, and 4 in MARC-145 cell. Total RNA was prepared and the mRNA levels of the syndecan genes were measured by real-time PCR. The relative copy number was presented. (B) MARC-145 cells were transfected with a control siRNA or siSDC4. After 48 h post transfection, cells were infected with PRRSV (MOI = 1) for 1 h at 4 °C. PRRSV attachment and SDC4 knockdown efficiency were analyzed by qRT-PCR. (C, D, E) MARC-145 cells were transfected with pCAGGS-HA vector (control) and pCAGGS-HA-syndecan 4 (HA-Sdc4). At 24 h post-transfection cells were infected with PRRSV (MOI = 1) for 1 h at 4 °C. (C) The virus attachment was examined by qRT-PCR. (D) The cells were infected with PRRSV (MOI = 1, 1 h) at 4 °C before incubated at 37 °C (1 h). Internalized virus was detected by qRT-PCR. (E) The overexpression of HA-SDC4 was analyzed by western blot.

incubated at 37 °C. The cells were fixed with 4% paraformaldehyde for 15 min after washing. The cells were then blocked with 1% bovine serum albumin, followed incubated with primary antibody and Alexa Fluor-conjugated secondary antibody. Unless otherwise specified, staining was performed at room temperature for 1 h. The nuclei were stained with DAPI. Images were captured by immunofluorescence microscopy or confocal microscopy.

2.8. Statistical analysis

All experiments presented were repeated three times. The data represented the mean $\pm$ SD. All data was analyzed by one-way ANOVA using the SPSS 17.0 software package (version 17.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Syndecan-4 involves in PRRSV attachment and entry

PRRSV infection in MARC-145 cells is more sensitive to heparin inhibition than its infection in PAM cells, implying that a heparin-like molecule plays a more predominant role in virus entry into MARC-145 cells than that in PAM [10]. Heparan sulfate glycosaminoglycans (HSPGs) act as the attachment factor for many viruses [17–20]. HSPGs on the macrophage surface have been suggested as PRRSV attachment factor which is beneficial for the concentration of the virions on the cell surface, therefore ensuring a more efficient infection [21,22]. Syndecans are the only HSPG that penetrates the cytoplasm and interacts with the cytoskeleton [14]. Therefore, we hypothesized that syndecans might be the main heparan sulfate

proteoglycan used for PRRSV attachment in MARC-145 cells.

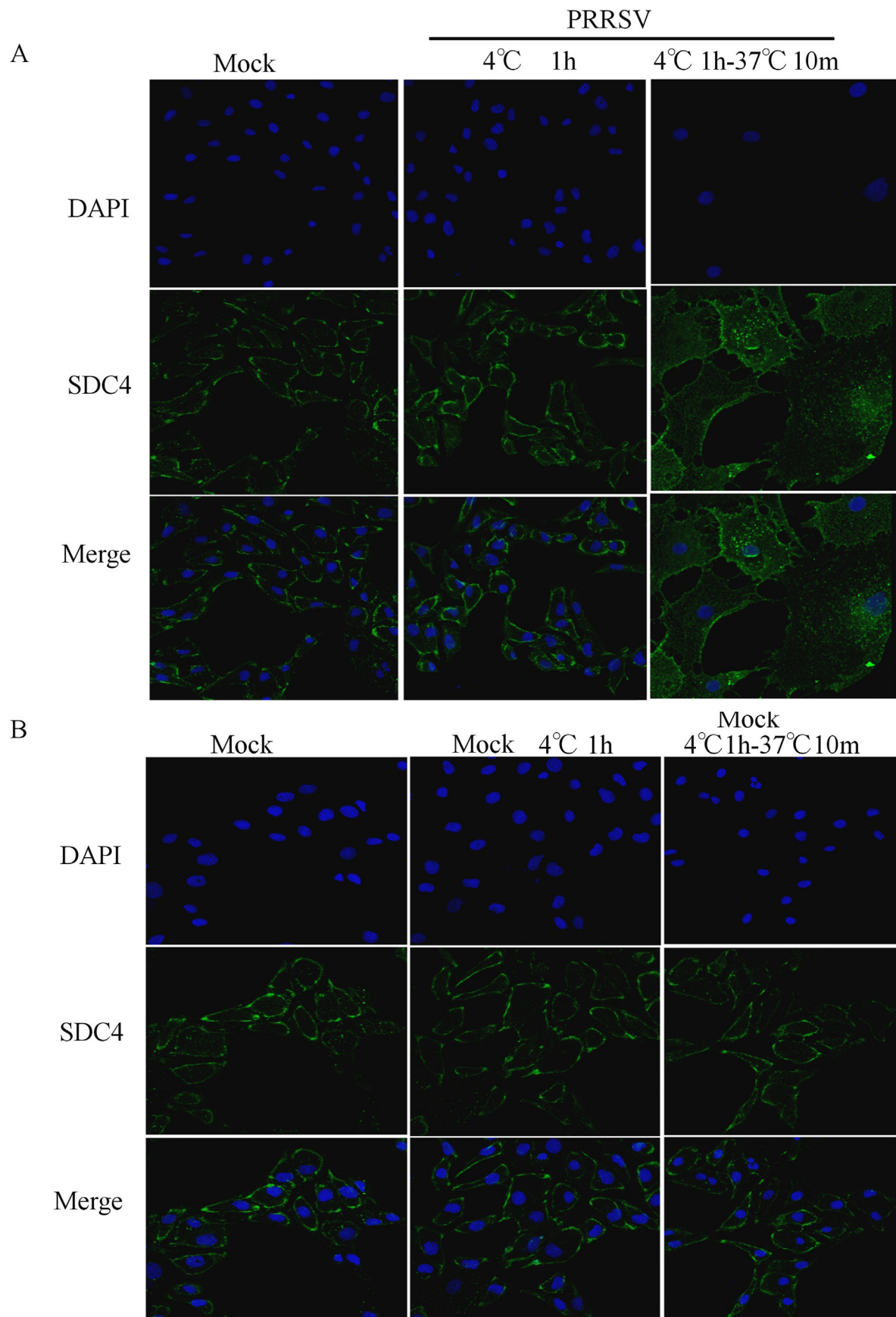
We examined the expression of syndecans in MARC-145 cells. The qRT-PCR result showed that syndecan-4 is most expressed one among syndecan1–4 (Fig. 1A). The PRRSV attachment was further assayed in SDC4 knockdown cells. As expected, knockdown of SDC4 significantly abolished PRRSV attachment compared to the control (Fig. 1B), suggesting SDC4 might function in PRRSV attachment. Moreover, virus attachment was examined in MARC-145 cells that over-expressed SDC4 (HA-SDC4). As expected, the result showed that virus attachment was significantly enhanced in SDC4 over-expressing cells (Fig. 1C).

We next determine whether the concentration of virus on the cell surface increased virus entry. We detected PRRSV entry in SDC4 over-expressing cells. The qRT-PCR result showed that PRRSV entry was also markedly increased (Fig. 1D). The overexpression of SDC4 was confirmed by western blot analysis (Fig. 1E).

3.2. PRRSV entry mediates syndecan-4 internalization

To further examine the functional role of SDC4 in PRRSV entry, we detected the cellular localization of SDC4 during PRRSV entry. We found that SDC4 was localized at the plasma membrane in mock-infected cells and the cells incubated with PRRSV at 4 °C. SDC4 was internalized into the cells when the temperature was shifted to 37 °C, which was the optimal temperature for PRRSV entry (Fig. 2A). To eliminate the possibility that the endocytosis of SDC4 was caused by the temperature change, we also determined the localization of SDC4 in cells cultured in DMEM medium at 37 °C. The immunofluorescence result showed that SDC4 resided at the plasma membrane (Fig. 2B), suggesting that temperature change

Fig. 2. Serum-starved cells were infected PRRSV (MOI = 1) at 4 °C for 1 h, or followed by the incubation for 10 min at 37 °C. After washed with PBS, cells were fixed in 4% paraformaldehyde for 15 min. The samples were blocked in 1% bovine serum albumin and incubated with syndecan-4 antibody for 2 h and Alexa Fluor[®] 488 goat anti-mouse IgG (H + L) for 1 h. The nuclei were stained by DAPI. Images were captured with cells by confocal microscopy.



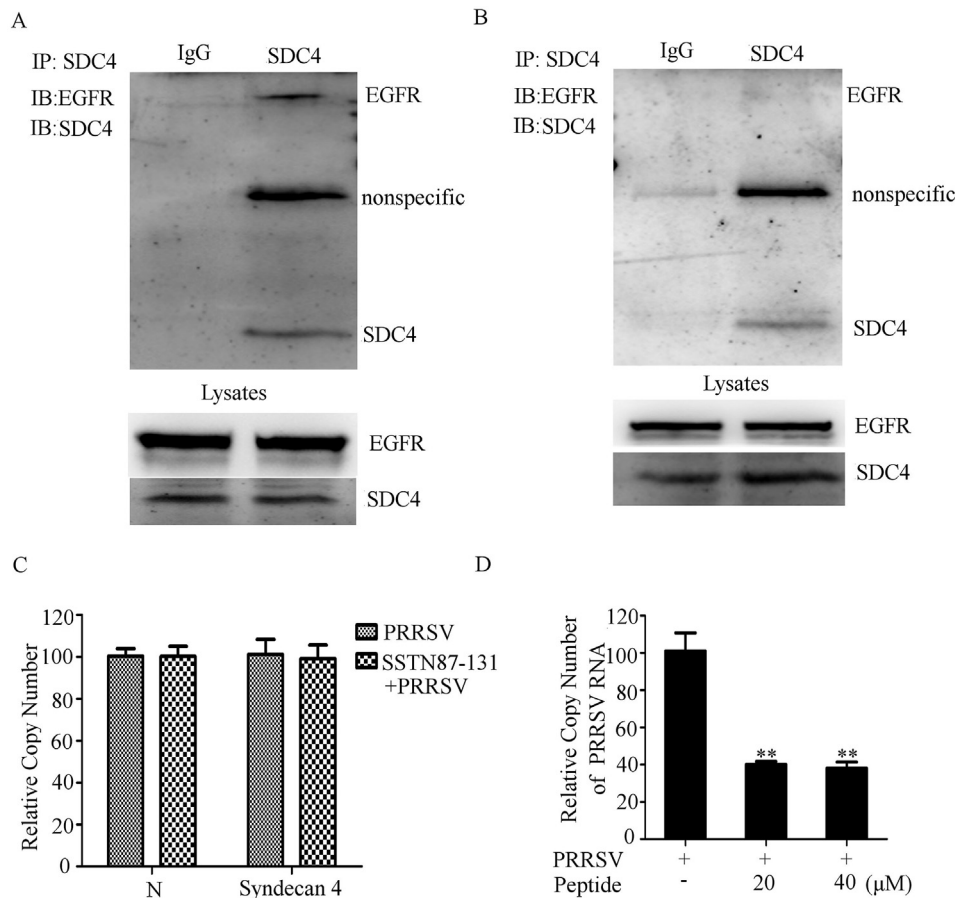


Fig. 3. Syndecan-4 mediates PRRSV entry by interacting with EGFR. (A) MARC-145 cells were lysed. The protein samples were subjected to SDS-PAGE and transferred to PVDF membrane. Anti-EGFR or anti-SDC4 monoclonal antibodies were used to detect EGFR or SDC4. (B) MARC-145 cells were pretreated with SSTN87-131 for 2 h and were lysed for co-immunoprecipitation assay. (C, D) The effects of SSTN87-131 on PRRSV entry and attachment. (C) Serum-starved MARC-145 cells were pretreated with SSTN87-131 for 1 h at 4 °C, followed incubated with PRRSV (MOI = 1) for 1 h at 4 °C with 40 μM SSTN87-131. PRRSV attachment was analyzed by quantitative real-time PCR. (D) Serum-starved MARC-145 cells were pretreated with different concentration of SSTN87-131 for 1 h, followed by incubation with PRRSV (MOI = 1, 1 h, 4 °C) with SSTN87-131. The cells were then incubated at 37 °C for 1 h in the presence with SSTN87-131. PRRSV entry was analyzed by qRT-PCR.

did not affect the localization of SDC4.

3.3. Syndecan-4 mediates PRRSV entry by interacting with EGFR

EGFR can be captured by the extracellular domain of SDC4 at residue 87–131 [15]. To address this in MARC-145 cells, we first performed co-immunoprecipitation assay. As expected, SDC4 was able to be immunoprecipitated with EGFR in MARC-145 cells (Fig. 3A). We next determined whether the interaction between SDC4 and EGFR was necessary for PRRSV entry. Peptides corresponding to residues at 87–131 in SDC4 (synstatin, SSTN87-131) was synthesized. The effect of SSTN87-131 on the interaction between SDC4 and EGFR was determined. The co-IP experiment showed that the interaction between SDC4 and EGFR could be disrupted by SSTN87-131 treatment (Fig. 3B). Next, we treated the cells with SSTN87-131 to determine its inhibitory effect on PRRSV entry. The result showed that PRRSV entry was significantly abolished in MARC-145 cells by SSTN87-131 treatment (Fig. 3D) while virus attachment was not affected by SSTN87-131 (Fig. 3C).

3.4. EGFR mediates the endocytosis of syndecan-4 during PRRSV entry

Our above result showed the interaction between SDC4 and EGFR played a crucial role in PRRSV entry. We next analyzed the

effect of EGFR activity on SDC4 endocytosis mediated by virus entry. The immunofluorescence assay showed that SDC4 in uninfected cells was mainly localized on the cell membrane (Fig. 4A), virus infection promoted its internalization (Fig. 4B). EGFR inhibitor treatment prevented the endocytosis of SDC4 during virus infection (Fig. 4C). Meanwhile, SSTN87-131 treatment also inhibited SDC4 endocytosis in a dose-dependent manner (Fig. 4 D and E). We, therefore, concluded that EGFR activation and its interaction with SDC4 mediated the endocytosis of SDC4 and PRRSV.

4. Discussion

Virus attachment to the plasma membrane and subsequent entry into host cells are crucial steps in virus infection. Heparan sulfate glycosaminoglycans can act as the attachment factor of various viruses [17–20]. Heparan sulfate glycosaminoglycans on the macrophage surface have been suggested as PRRSV attachment factor [21,22]. Though treatment of PAMs with heparin or heparinase significantly inhibited PRRSV infection, the attachment of PRRSV could not be entirely blocked, suggesting the presence of other factors on the cell surface that are responsible for virus attachment. Sialoadhesin (Sn) mediated PRRSV attachment and internalization in PAMs. Meanwhile, CD163 is associated with the uncoating of virus particles. Sn is expressed on the cell surface of PAMs, but not in MARC-145. Therefore, we suspected that other

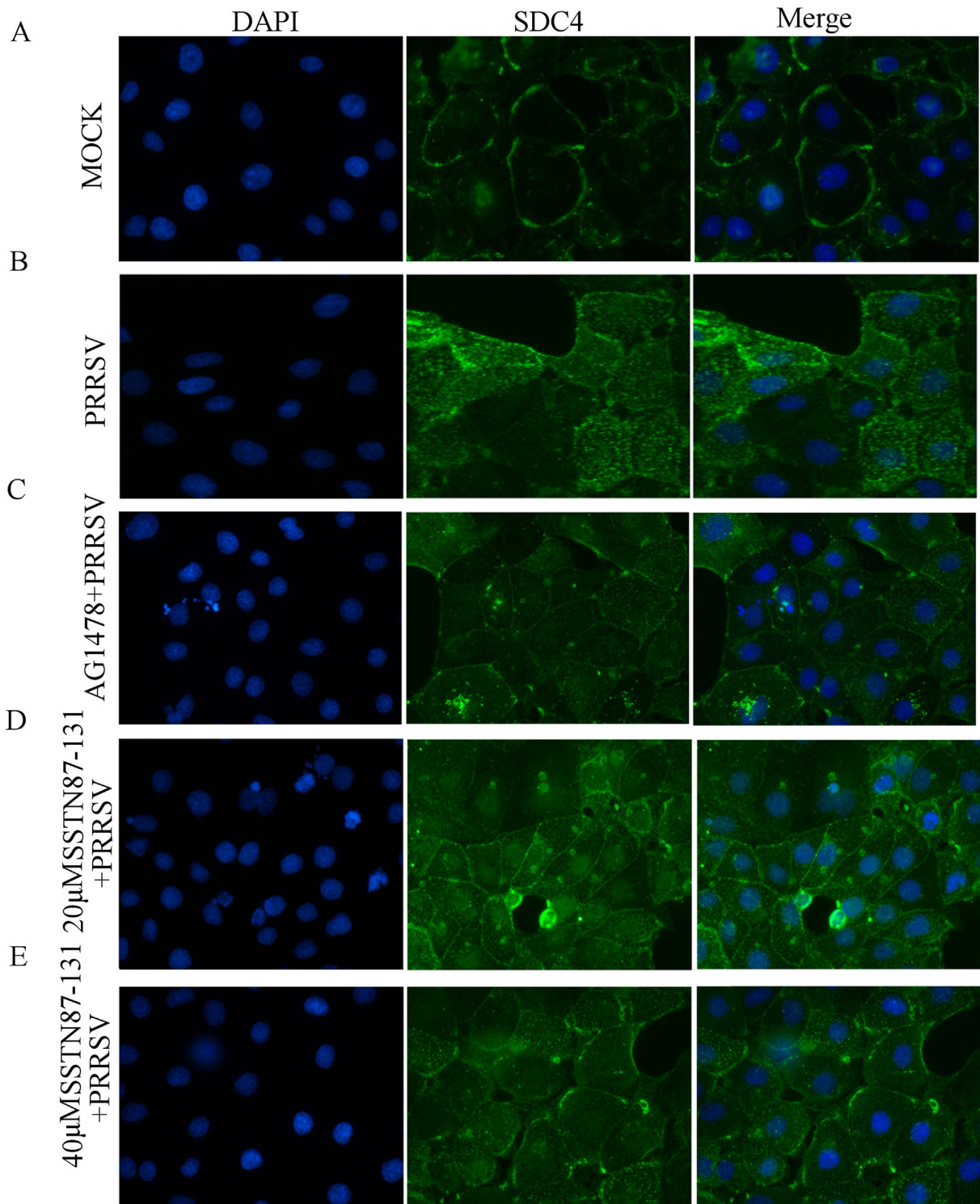


Fig. 4. Serum-starved cells were infected PRRSV (MOI = 1) at 4 °C for 1 h (A), or followed by the incubation for 10 m at 37 °C (B). Serum-starved cells pretreated with AG1478 (C) or SSTN87-131 (20 μM or 40 μM) (D, E) were first infected with PRRSV (MOI = 1) for 1 h at 4 °C and then incubated for 10 m at 37 °C in the presence of AG1478 or SSTN87-131. After washed with PBS, the cells were fixed and stained as described in method. Images were captured with cells by immunofluorescence microscopy.

cellular factors could mediate PRRSV attachment to MARC-145 cells. SDC4 is one of four transmembrane heparan sulfate proteoglycans, whose extracellular domains interact with various soluble and insoluble factors in the extracellular matrix (ECM). SDC4 mediates numerous cellular processes through signaling pathways that connected with cellular proliferation, migration, mechanotransduction, and endocytosis. In our study, we found that

SDC4 played an important role in PRRSV attachment and entry to MARC-145 cells. Knockdown of SDC4 significantly impaired PRRSV attachment, whereas overexpression of SDC4 enhanced PRRSV attachment and internalization.

Cell-adhesion events activate some signaling pathways that lead to the formation of focal adhesions and actin stress fibers in cells plated on fibronectin. The cell-surface heparan sulfate

glycosaminoglycans are required in these processes because heparinase treatment prevented the formation of focal adhesions and stress fibers. SDC4 and integrins are the primary transmembrane receptors of focal adhesions in cells adherent to extracellular matrix molecules [23–26]. Moreover, they act cooperatively in modulating cell spreading, the assembly of focal adhesions, and actin stress fiber formation [27–30]. Studies demonstrated that SDC4 mediated signaling events parallel to integrins [31], SDC4 and integrins connect with paxillin through the different adaptors, leading to the activation of FAK and Src, which then initiates the signaling cascade to promote focal adhesion and stress fiber formation [32,33]. Protein kinase C (PKC) belongs to a family of serine/threonine kinases, which is also associated with focal adhesions. It reported that PKC δ is activated during PRRSV infection and plays an important role at the early steps of virus infection [34]. PKC activation directs the localization of SDC4 to focal adhesion sites [23]. The role of the interaction between SDC4 and PKC during PRRSV entry awaits further investigation.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that belongs to the ErbB family. It is a central player in cellular signaling events that control a broad range of processes [35]. Several studies have demonstrated that EGFR is a critical coreceptor for hepatitis C virus, influenza A virus, and human cytomegalovirus entry [36–39]. A new study revealed that SDC1 and SDC4 capture EGFR family members and integrin $\alpha\beta 1$ with the ectodomain as the binding sites [15]. We determined that SDC4 interacted with EGFR in MARC-145 cells. Moreover, we revealed that SDC4 peptide treatment not only abolished the interaction between SDC4 and EGFR but also inhibited PRRSV entry. At the same time, virus attachment was not affected by peptide treatment.

In summary, our studies demonstrated that during PRRSV infection in MARC-145 cells, the virus first attached heparin sulfate motifs on SDC4 through viral proteins GP5 and M [11,12]. The interaction triggered the activation of EGFR through a currently unknown mechanism, which in turn might activate the downstream signal pathways to promote virus entry. One study demonstrates that EGFR-PI3K signaling controls cofilin activity to facilitate herpes simplex virus 1 entry into neuronal cells [40]. Similar to this, a newly published paper suggests EGFR regulates cofilin activity and promotes transmissible gastroenteritis virus entry into intestinal epithelial cells [41]. Whether EGFR-PI3K-cofilin pathway is involved in PRRSV entry need further investigation. Nevertheless, our findings presented here extended our current understanding of PRRSV entry mechanism and may help the development of novel strategies for treatment of PRRSV infection.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (A0201200499).

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2016.05.084>.

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