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The involvement of FAK-PI3K-AKT-Rac1 pathway in porcine reproductive and respiratory syndrome virus entry

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ABSTRACT

CD163 and sialoadhesin had been reported as the two receptors for porcine reproductive and respiratory syndrome virus (PRRSV) infection. The signaling pathway activated by PRRSV entry was seldom reported. In our studies, we demonstrated that PRRSV entry triggers FAK, PI3K, AKT and Rac1 activation. The signaling pathway FAK-PI3K-AKT-Rac1 is essential for PRRSV entry. Blocking FAK by PF573228 attenuates the activation of PI3K, AKT, Rac1 and the cytoskeleton remodeling induced by virus entry. Inhibitors to FAK, PI3K, AKT and Rac1 can significantly inhibit the virus entry. In conclusion, our observations reveal that PRRSV triggers the activation of FAK-PI3K-AKT-Rac1 signaling pathway to facilitate its entry into cells.

1. Introduction

PRRSV infection causes severe respiratory symptoms and reproductive failure, resulting in significant loss in swine industry worldwide [1,2]. Most of pathogenesis researches for PRRSV were focused on immune response. Despite years of thorough research, no efficient treatment is available for PRRSV infection. The host factors that are utilized by PRRSV in the different infection stages, such as entry, replication, assembly, release, are still poorly understood.

The PI3Ks, which can be divided into three classes, are a family of lipid kinases that regulates variety kinds of cellular activities [3–8]. AKT acts as the one of the key downstream effectors of PI3K to regulate cell activities [9–11].

Before PI3K involvement in signal transduction, the signal must be sensed by cell surface receptors. The assembly of integrins induces aggregation of some signaling molecules to form focal adhesions (FAs) [12]. These signaling molecules include FAK, SRC, p130Cas, talin and paxillin [13]. FAK is a tyrosine kinase that can be recruited to focal adhesion and activated after integrin binds to the ligand [14–16]. In some cell types, FAK associates with PI3K [17,18]. FAK can phosphorylate the p85 subunit of PI3K *in vitro*, indicating that PI3K might be a FAK effector. The phosphorylation of p85 subunit can subsequently activate PI3K-AKT signal pathway [19–21].

Virus regulates vesicular trafficking through actin remodeling [22] and Rac1 is an important regulator of this process. During virus entry, Rac1 functions as the effector to polymerize the actin cytoskeleton to provide the mechanical force necessary for virion surfing, receptor clustering, virus-containing vesicles internalization [23,24]. In some cases, PI3Ks act at the upstream of Rac1 to induce actin remodeling and endocytosis [25–29].

Here we revealed that FAK-PI3K-AKT is crucial for PRRSV entry process. Rac1 acts as a key downstream effector of FAK-PI3K-AKT pathway in regulating PRRSV entry.

2. Methods and materials

2.1. Cells and virus

MARC-145 cells or PAM cells that stably expresses CD-163 were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) or RPMI-1640 (Hyclone, USA.) supplemented with 10% fetal

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bovine serum (FBS, Gibico, USA.), penicillin (100 U/ml) and streptomycin (100 μg/ml). PRRSV (JXA1 strain) was expanded by growth in MARC-145 cells. Virus was purified, titered and stored at −80 °C.

2.2. Chemicals, antibodies and other reagents

The FAK inhibitor PF-573228 and AKT Inhibitor VIII were obtained from Sigma–Aldrich. Antibodies against p-AKT(Ser473), AKT, p-FAK(Tyr397), FAK, were purchased from Cell Signaling Technology (U.S.A.). The monoclonal antibody against PRRSV nucleocapsid protein was preserved in our laboratory. PI3K inhibitor LY294002 and Rac1 inhibitor NSC23766 were obtained from Santa Cruz (U.S.A.). All reagents were stored at −20 °C in single use aliquots.

2.3. Virus entry assay

Virus entry was determined as described previously [30]. Briefly, serum starved cells were pretreated with various inhibitors for 2 h at 37 °C and then incubated at 4 °C with PRRSV for 1 h to allow virus binding. Cells were washed and transferred to 37 °C for 40 min. The cells were washed 3 times with citric acid buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) to inactivate cell surface virions. The cells were then washed three times with PBS to remove the acidic buffer. The internalized virus was detected by both western blot and real-time PCR.

2.4. Expression and purification of GST-PAK-PBD

The expression plasmids of pCold-GST-PAK-PBD was transformed into *Escherichia coli* expression strain BL-21-Codon plus (DE3)-Rosetta 2 cells. 5 ml of LB broth containing 100 μg/ml ampicillin was inoculated with a single colony and incubated overnight at 37 °C. The following day, the culture was used to inoculate 500 ml of LB broth and 100 μM of IPTG was added when OD_{600} reached 0.8. After 18 h of incubation at 20 °C, the cells were harvested by centrifugation at 8000 g for 15 min and resuspended in Tris buffer (300 mM NaCl, 40 mM Tris–HCl, 10% glycerol, 0.1 mM PMSF). Resuspended cells were sonicated on ice and then centrifuged at 14000 g for 20 min. GST agrose beads was incubated with the supernatant at 4 °C for 2 h and then washed three times with washing buffer (50 mM Tris-Cl pH 7.5, 0.5% (w/v) Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF). The washed GST-PAK-PBD beads were kept at 4 °C.

2.5. Pull-down assay for Rac1-GTP

Serum-starved MARC-145 cells were incubated with PRRSV at 4 °C for 1 h and then the cells were washed and transferred to 37 °C. At desired times, cells were washed three times with ice-cold PBS and lysed in 700 μl of lysis buffer (50 mM Tris–HCl pH 7.2, 1% (w/v) Triton X-100, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.2 mM PMSF). The lysates were centrifuge at 14000 g for 15 min. Equal amount supernatant was incubated with the GST-PAK-PBD beads mentioned above for 2 h at 4 °C and subsequently washed three times with washing buffer. The Rac1-GTP was detected by western blot with Rac1 antibody.

2.6. Virus labeling

Purified PRRSV resuspended in HNE buffer were incubated with DiD (8 μM)for 60 min at room temperature. The labeled virions are purified through Sephadex G-50 column previously equilibrated by HNE buffer to remove the extra dye. The titers of different fractions were assayed before stored at −80 °C.

2.7. Confocal fluorescence microscopy

MARC-145 cells seeded on coverslips were first serum starved. DiD labeled PRRSV was incubated with the cells in serum free DMEM for 1 h at 4 °C. Cells were washed three times with PBS and then transferred to 37 °C. Cells were fixed with 4% formaldehyde at different time post infection, permeabilized with 0.3% Tween-20 and incubated with 5% BSA for 30 min, the F-actin is labeled with FITC conjugated phalloidin. The nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI, Dinguo, China).

2.8. Western blot analysis

The harvested cells were lysed in RIPA buffer (Tris–HCl 50 mM pH7.4, 1 mM EDTA, 150 mM NaCl, 1% NP40, 5 mM NaF, 0.25% sodium deoxycholate, 5 mM sodium orthovanadate, 0.4% SDS, 0.1 mM PMSF) containing protease inhibitor, and phosphotase inhibitor (Thermo, USA) for 1 h at 4 °C. The lysate was sonicated on ice and centrifuged at 14000 g for 15 min at 4 °C. The protein concentration was determined by BCA protein assay kit (Thermo, USA). Equal amount of protein were separated on SDS-PAGE and then transferred to PVDF membrane. The membrane was blocked with 5% nonfat milk or 5% BSA for 1 h at room temperature. Membrane was washed with PBST and incubated with primary antibody at 4 °C overnight and the secondary antibody for 1 h. The immunoreactive bands were detected by enhanced chemiluminescence system (Vazyme, China).

2.9. Plasmid constructs

To construct HA-tagged Rac1 and GST-PAK-PBD plasmids, the gene fragments were amplified by PCR using cDNA as the template with specific primers (http://www.sciencedirect.com/science/article/pii/S0166354213002477Table 1) and were cloned into pCAGGS or pCold-I-GST vector respectively. pCAGGS-Rac1-Q61L mutants were prepared with Mut Express® II Fast Mutagenesis kit(Vazyme, China) according to the manufacturer's instruction using pCAGGS-Rac1 as the template with specific primers (http://www.sciencedirect.com/science/article/pii/S0166354213002477Table 1).

2.10. Quantitative real-time PCR

Total RNA were extracted by Trizol and the reverse transcription was done with HiScript™ 1st Strand cDNA Synthesis Kit (Vazyme, CHINA). Quantitative real-time PCR was carried out with AceQ qPCR Probe Master Mix (Vazyme, China). The primer and probe sequences used are shown in Table 1.

2.11. Cytotoxicity assay

The MARC-145 or PAM cells were seeded in 96-well cell culture plate. After 24 h of incubation, medium was replaced with DMEM containing different concentrations of inhibitors. 24 h later, the cytotoxicity was measured with CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, USA) according to the manufacturer's instructions. All experiments were done with inhibitor concentration that do not affecting the cell viability.

2.12. Statistical analysis

Experiment was performed at least three times. The data was showed as means ± SD. Statistical analysis was determined by one-way ANOVA. The differences between means were considered significant * $p < 0.05$, very significant at ** $p < 0.01$.

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

Gene	Primer sequence(5′ to 3′)
pCold-I-Gst-PAK-PBD	Forward: CCACCATATGAAGAAAGAGAAAGAGC Reverse: GGCACTCGAGTTAAGCTGACTTATCTGTAA
pCAGGS-Rac1	Forward: GCTCAGGTACCATGCAGGCCATCAAGTGT Reverse: GCTCACTCGAGTTACAACAGCAGGCATTTTC
pCAGGS-Rac1-Q61L(Quikchange)	Forward:GGCTTATGGGATACAGCTGGACTCGAAGATTATGACAGATTACGC Reverse: GCGTAATCTGTCATAATCTTCGAGTCCAGCTGTATCCCATAAGCC
N	Forward: TTGTGTCTGTCGTCGATCCAG Reverse: AAACTCCACAGTGTAACTTATCCTC Probe: (FAM) CGCTGGAACTTGTGCCCTGTCA (Eclipse)
Actin	Forward: TGACTGACTACCTCATGAAGATCC Reverse: TCTCCTTAATGTCACGCACGATT Probe: (FAM) CGGCTACAGCTTCACCACCACGGC (Eclipse)

3. Results

3.1. PRRSV activates the FAK-PI3K-AKT signaling pathway during entry

The HP-PRRSV(strain HuN4) has been suggested to modulate the PI3K-AKT pathway during the early stage of PRRSV infection [31]. In our studies, we examined if PRRSV strain (JXA1) can also activate PI3K-AKT pathway in MARC-145 cells. The cells were harvested at 0–60 min post infection. Our western blot analysis showed the activation of AKT in PRRSV infected cells at 15–30 min. The phosphorylation of AKT gradually decreased from 45 min post infection (Fig. 1A) suggesting that PRRSV (strain JXA1) entry activated AKT.

Previous report showed that FAK functions at the upstream of PI3K-AKT pathway. Binding of the phosphopeptide containing Y397 and the adjacent residues of FAK stimulated PI3K activity *in vitro* [32]. FAK can phosphorylate p85 subunit of PI3K *in vitro*, indicating

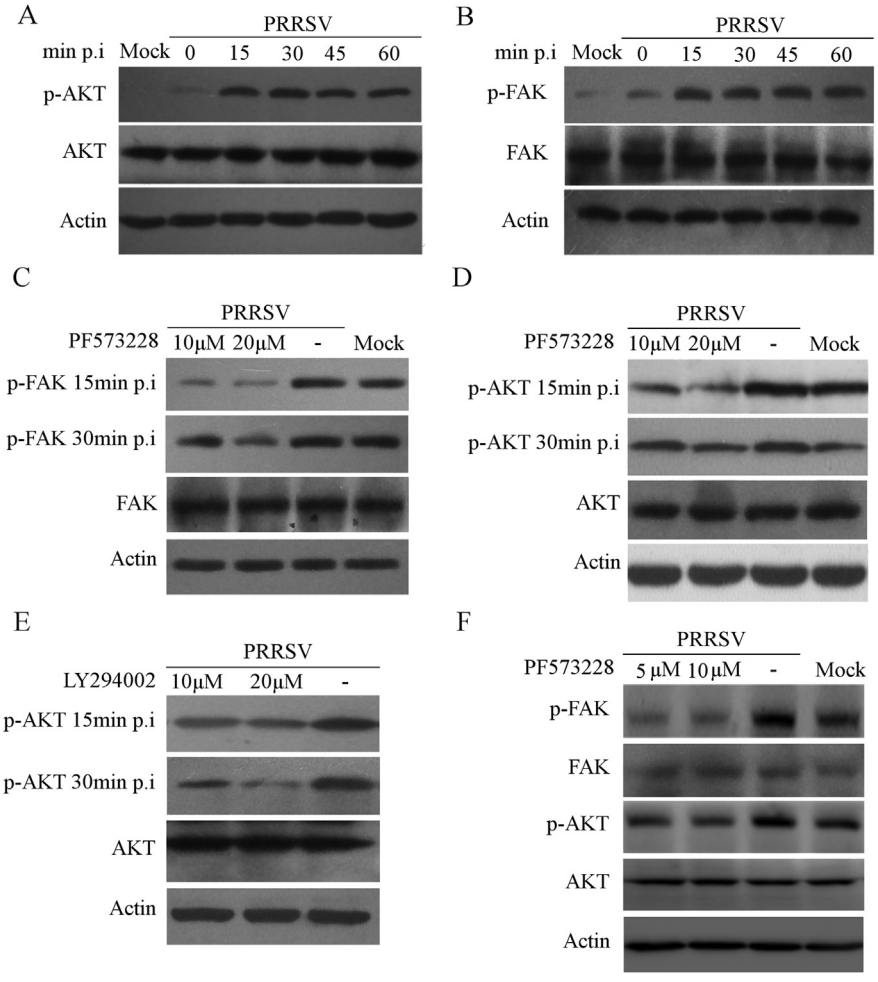

Fig. 1. Serum-starved MARC-145 cells were mock-infected or infected with PRRSV virus at a MOI of 1 at 4 °C for 1 h. Cells were washed and then transferred to 37 °C. The cells were harvested at 0–60 min p.i. Phosphorylation of (A)AKT, (B) FAK were analyzed by western blot. MARC-145 cells were serum starved and then treated with medium containing PF573228, LY294002 or DMSO. Cells were infected with the PRRSV at an MOI of 1 at 4 °C for 1 h, and then cells were washed and transferred to 37 °C. The cell were harvested at 15 and 30 min p.i. Phosphorylation of (C)FAK, (D) (E) AKT were analyzed by western blot. (F) Serum-starved PAM cells were treated with medium containing PF573228 or DMSO. Cells were infected with the PRRSV at an MOI of 1 at 4 °C for 1 h, and then cells were washed and transferred to 37 °C. The cell were harvested at 15 min p.i. Phosphorylation of FAK, AKT were analyzed by western blot.

that PI3K might be a FAK effector. It is shown that FAK phosphorylation occurs minutes after KSHV infection [33]. We next studied if the PRRSV entry induces FAK activation. As expected, FAK could be phosphorylated at 15–30 min, its activation then gradually decreased in the same way as the PI3K-AKT pathway(Fig. 1B). Taken together, FAK and PI3K-AKT can be activated during PRRSV entry.

We next examined whether PRRSV entry-induced FAK activation contributes to PI3K-AKT pathway activation. MARC-145 cells were pretreated with a potent and selective inhibitor of FAK (PF573228) before infected with PRRSV. The cells were collected at 15 min and 30 min p.i when the AKT and FAK were highly activated in the infected cells. As demonstrated in western blot analysis, PF573228 significantly reduced the phosphorylation of FAK and AKT at these two time points in a concentration dependent manner (Fig. 1C,D), indicating that the FAK acts at the upstream of PI3K-AKT signaling pathway. To ensure that the AKT was activated in a PI3K dependent manner during PRRSV entry as reported previously [31], we pretreated MARC-145 cells with the LY294002 (a specific inhibitor for PI3K) for 2 h before virus infection. The result showed that LY294002 significantly reduced the phosphorylation of AKT (Fig. 1E). We further confirmed the signaling pathway in PAM cells by inhibiting FAK. As expected, the FAK and AKT were also activated during PRRSV entry and the FAK inhibitor PF573228 significantly inhibited their activation(Fig. 1F). Taken together, our results suggested that PRRSV controls FAK-PI3K-AKT signaling pathway during its entry.

3.2. FAK-PI3K-AKT signaling pathway is required for PRRSV entry

To further determine if the activation of FAK-PI3K-Akt signaling pathway was necessary for PRRSV entry, we pretreated MARC-145 cells with FAK (PF573228), PI3K (LY294002) or Akt (Akt VIII) inhibitor before they were infected with PRRSV. The internalization of the virus was measured by real-time PCR. Our data showed that virus internalization at 40 min p.i. was inhibited by 46%, 54% and 41% respectively (Fig. 2A), which was confirmed by western blot analysis on the amount of viral nucleocapsid protein in cells at 40 min p.i. Pretreatment of the cells with PF573228, LY294002 or Akt VIII can significantly decrease the amount of N protein (Fig. 2B, C, D). Further study demonstrated that inhibition of FAK by PF573228 in PAM cells can also reduce PRRSV entry (Fig. 2E). Taken together, PRRSV entry requires the activation of FAK-PI3K-AKT signaling pathway.

3.3. Virus remodels the actin cytoskeleton by regulating FAK-PI3K-AKT signaling pathway

When PRRSV was attached to MARC-145 cells in the 4 °C and then transferred to 37 °C, rapid reorganization of F-actin network was observed from 15 min p.i., and the lamellipodia formed at the cell periphery (Fig. 3A) as white arrow pointed. FAK inhibitor (PF573228) treatment significantly reduced the PRRSV induced lamellipodia (Fig. 3B) and the PRRSV entry. Our observation suggested that virus entry remodeled the actin cytoskeleton by controlling the FAK-PI3K-Akt pathway.

3.4. Rac1 acts as the downstream effector of FAK-PI3K-AKT signaling pathway

Previous reports suggested that Rac1 activation can induce the lamellipodia formation by regulating actin polymerization [34,35]. Since PRRSV entry induced the lamellipodia formation, we next

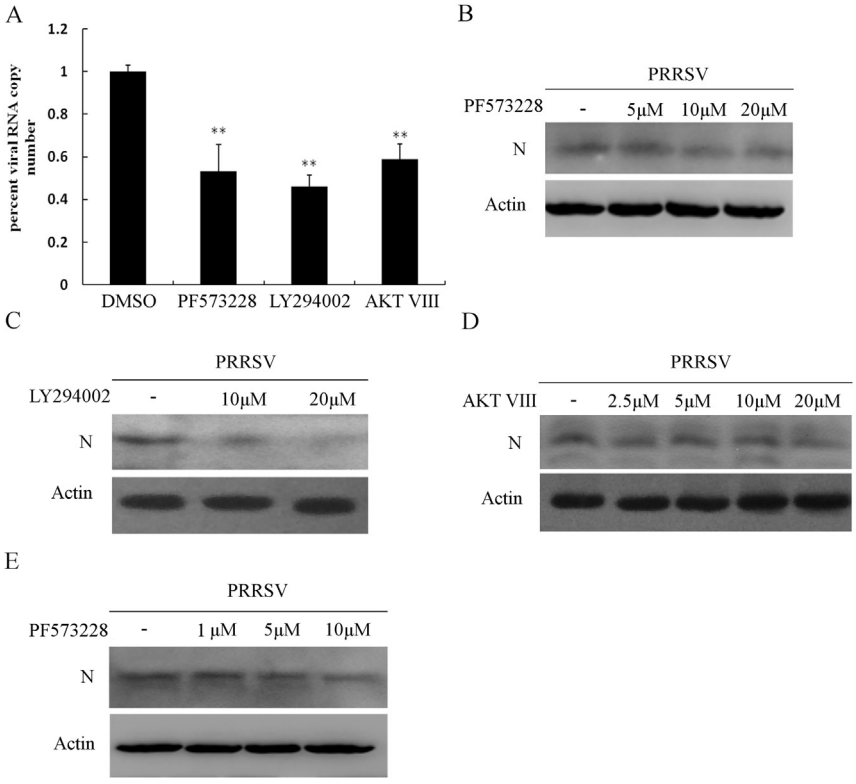

Fig. 2. FAK-PI3K-AKT signaling pathway is required for PRRSV entry. MARC-145 cells were serum starved and then treated with medium containing PF573228, LY294002,AKT VIII or DMSO. Cells were infected with the PRRSV at an MOI of 1 at 4 °C for 1 h, and then cells were washed and transferred to 37 °C for 40 min. The cells were washed 3 times with citric acid buffer to inactivate cell surface virions and washed two times with PBS to remove the acidic buffer. (A)The internalized virus was detected by real-time PCR. (B) (C) (D)These results were also confirmed by western blot analysis. (E) PAM cells were treated with PF573228 and the virus entry was measured as mentioned above.

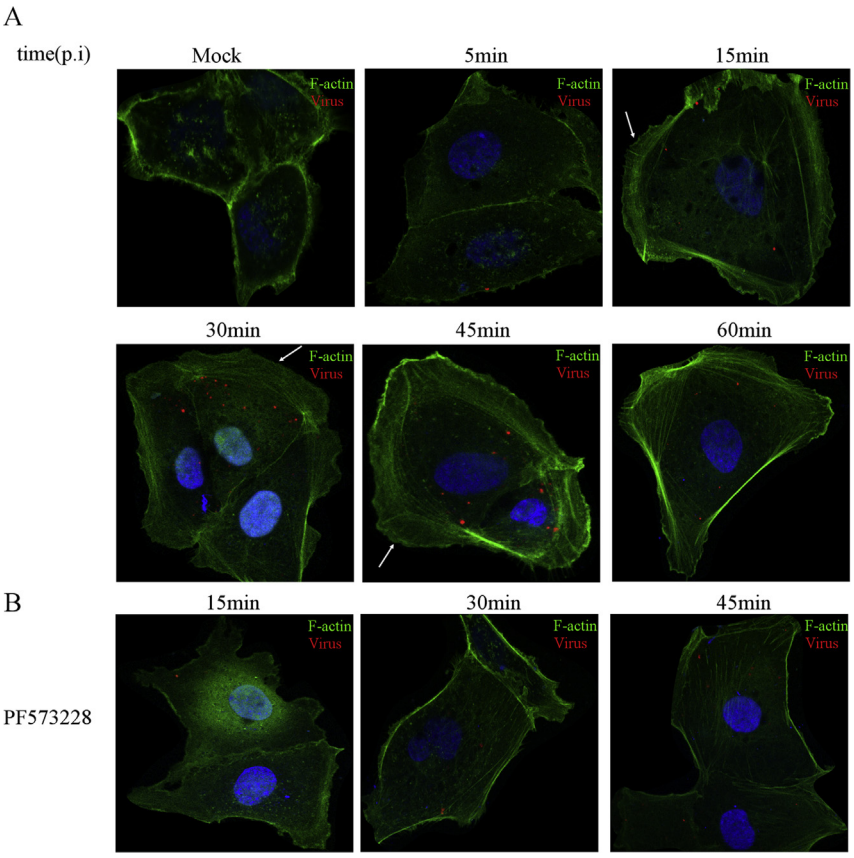

Fig. 3. Virus remodels the actin cytoskeleton to facilitate its entry by regulating FAK-PI3K-AKT-Rac1 signaling Pathway. DMSO treated (A) or PF573228 treated (B) serum-starved MARC-145 cells were infected with DiD labeled PRRSV virus at a MOI of 10 at 4°Cfor 1 h, and then cells were washed and transferred to 37 °C. The cells were fixed with 4% formaldehyde and permeabilized with 0.3% Tween-20 at different time post infection. The F-actin is stained with FITC conjugated phalloidin. The nuclei were stained with 4′-6-diamidino-2-phenylindole.

tested if the PRRSV entry activates Rac1. MARC-145 cells were infected for 0–60 min, the cell lysate was harvested for Rac1-GTP pull-down assay. Our data showed that the small GTPase Rac1 was activated at 15–30 min p.i and the activation gradually decreased from 45 min p.i., implicating that PRRSV entry activated Rac1(Fig. 4A). To ensure that PRRSV entry is related to Rac1 activation, a specific inhibitor to Rac1 (NSC23766) was used and PRRSV entry was significantly inhibited (Fig. 4B, C).

These findings suggested that PRRSV needs Rac1 function in entry. Previous report had shown that PI3Ks can act at the upstream of Rac1 to induce actin remodeling and endocytosis. So we wandered if the Rac1 acts as the downstream effector during PRRSV entry. To prove this, PRRSV infected cells were treated with the inhibitors of FAK or PI3K, the Rac1 activation levels were tested by western blot analysis. The result showed that these inhibitors significantly inhibited the PRRSV induced Rac1 activation (Fig. 4D). Furthermore, the inhibitory effect of AKT inhibitor on PRRSV entry was significantly reversed in MARC-145 cells that transiently expressed constitutively active Rac1 (Rac1-Q61L) (Fig. 4E, F). Taken together, our results suggested that FAK-PI3K-AKT functions at the upstream of Rac1.

4. Discussion

In the studies, we focused on the signaling pathways involved during PRRSV entry into the cell. We analyzed several important signaling molecules that are commonly involved in the entry of a variety of viruses. We found that PRRSV entry activates FAK-PI3K-AKT-Rac1 pathway, resulting in the remodeling of the microfilament cytoskeleton to facilitate virus entry.

Our results showed that PRRSV infection activate PI3K-AKT at early stage of infection, which is consistent with the previous report [31]. We further determined that inhibition of PI3K-AKT pathway by specific inhibitor targeting PI3K or AKT can dramatically decrease PRRSV internalization.

It was shown previously that FAK, the molecule necessary for the outside-in signal transduction by integrins, associates with PI3K upon integrin activation in some cell types, which suggests that PI3K might be a FAK effector [17,18]. We next examined the relationship between FAK and PI3K during PRRSV entry. We showed that FAK was dramatically activated by PRRSV from 15 min p.i. Meanwhile, the inhibition of FAK by specific inhibitor PF573228 reduced PRRSV induced phosphorylation of FAK and AKT. Furthermore, FAK inhibitor prevented PRRSV internalization just like the PI3K or AKT inhibitor did. Recently, it was reported that FAK is activated in PI3K-AKT dependent manner [36]. We excluded this possibility by examining if the PI3K inhibitor can inhibit FAK activation during PRRSV entry. Our result showed that LY294002 did not reduce FAK phosphorylation (data not shown). These data suggested that FAK may act as the upstream of PI3K-AKT pathway and FAK-PI3K-AKT signaling pathway controls the entry process of PRRSV.

In most cases, the PI3K-AKT pathway control virus entry by stimulating the remodeling of actin filaments [37,38]. We speculated

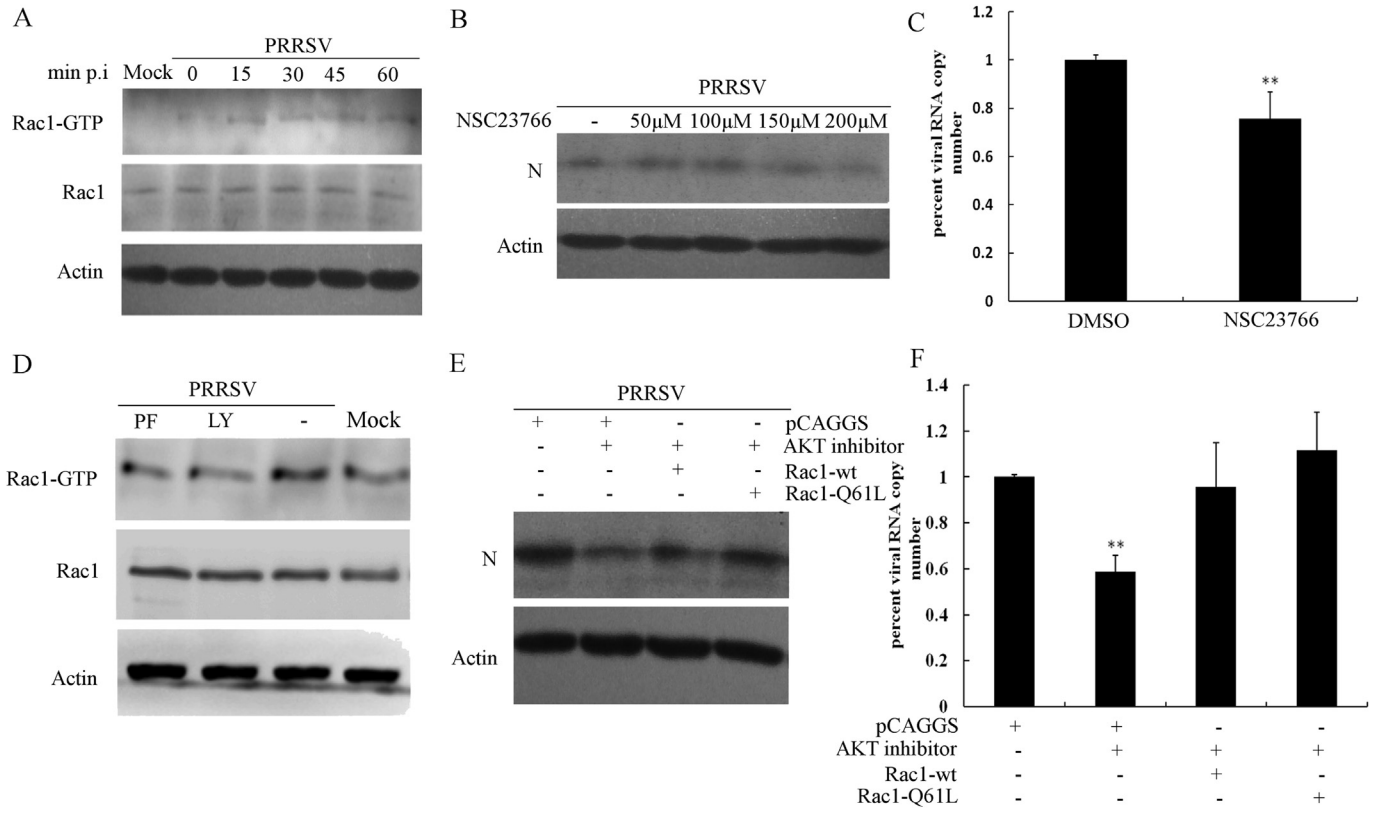

Fig. 4. Rac1 acts as the downstream effector of FAK-PI3K-AKT signaling pathway (A) PRRSV entry induces activation of Rac1 GTPase. Serum-starved cells were mock treated with DMEM or infected with PRRSV virus (MOI = 1) at 4°Cfor 1 h. The cells were washed and transferred to 37 °C. The cells were harvested at 0–60 min p.i. The lysates were centrifuged and the GST agrose beads binding PAK-PBD incubated with equal amounts of supernatant protein for 2 h at 4 °C. Rac1-GTP was detected by western blot (B), (C) PRRSV entry depends on Rac1. MARC-145 cells were serum starved and then treated with medium containing NSC23766 or DMSO. Virus internalization was monitored by both western blot and real-time PCR. (D) FAK, PI3K controls Rac1 activation. LY294002, PF573228 and DMSO treated cells were infected with PRRSV and collected at 15 min p.i. Rac1 activation was analyzed by Rac1-GTP pull down assay. (E) (F) Rac1 is a downstream effector of AKT. Cells transiently transfected with pCAGGS plasmid or plasmid express Rac1 were serum starved for 12 h, and treated with AKT VIII for 2 h. The cells were then infected with PRRSV, internalized virus was detected by both western blot analysis and real-time PCR.

that PRRSV regulates the FAK-PI3K-AKT pathway to control actin remodeling. Our results showed PRRSV induced actin cytoskeleton reorganization and lamellipodia formation during the early infection, which could be inhibited by the treatment of FAK inhibitor. It had been reported that Rac1 was responsible for lamellipodia formation. Our studies confirmed that Rac1 was activated during PRRSV entry and it acted as a downstream effector of FAK-PI3K-AKT signaling pathway.

Taking our observations together, FAK-PI3K-AKT-Rac1 signaling pathway activated by PRRSV leads to reorganization of actin cytoskeleton which is beneficial for virus internalization.

Conflict of interest

None.

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Transparency document

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