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ORIGINAL ARTICLE

Receptor-bound porcine epidemic diarrhea virus spike protein cleaved by trypsin induces membrane fusion

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Abstract Porcine epidemic diarrhea virus (PEDV) infection in Vero cells is facilitated by trypsin through an undefined mechanism. The present study describes the mode of action of trypsin in enhancing PEDV infection in Vero cells during different stage of the virus life cycle. During the viral entry stage, trypsin increased the penetration of Vero-cell-attached PEDV by approximately twofold. However, trypsin treatment of viruses before receptor binding did not enhance infectivity, indicating that receptor binding is essentially required for trypsin-mediated entry upon PEDV infection. Trypsin treatment during the budding stage of virus infection induces an obvious cytopathic effect in infected cells. Furthermore, we also show that the PEDV spike (S) glycoprotein is cleaved by trypsin in virions that are bound to the receptor, but not in free virions. These findings indicate that trypsin affects only cell-attached PEDV and increases infectivity and syncytium formation in PEDV-infected Vero cells by cleavage of the PEDV S protein. These findings strongly suggest that the PEDV S protein may undergo a conformational change after receptor binding and cleavage by exogenous trypsin, which induces membrane fusion.

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Introduction

Porcine epidemic diarrhea virus (PEDV), a member of the family *Coronaviridae*, is an economically important pathogen of swine. PEDV causes acute watery diarrhea, resulting in approximately 50% mortality among suckling piglets and reduced weight among fattening pigs [10]. Although the structural and pathological properties of PEDV are similar to those of other group 1 coronavirus, including human coronavirus 229E (HCoV-229E), transmissible gastroenteritis virus (TGEV) and feline infectious peritonitis virus, many biological issues, such as the role of trypsin in infection, remain unresolved [7, 12, 36]. The first successful propagation of PEDV in cell culture was done by supplementing the Vero cell culture medium with trypsin [15]. The addition of trypsin was shown to induce fusion of the infected Vero cells, resulting in the formation of multiple syncytia, and produced a significant increase in virus titer after several passages. Soon afterwards, several other groups performed PEDV infection in vitro using the same conditions and reported similar findings [20, 21]. On the other hand, another study reported the successful propagation of the P-5 V strain in porcine enterocyte cell lines without trypsin supplementation of the medium, suggesting that the proteolytic processing of PEDV in enterocytes may have occurred during maturation or prior to virus release [17].

The spike (S) glycoprotein is the dominant surface protein in coronaviruses. The protein is responsible for virus attachment and fusion. The requirement of protease-cleaved S glycoprotein has been reported for almost all group 2 and 3 coronavirus. For example, infection by severe acute respiratory syndrome coronavirus (SARS-CoV) and murine hepatitis virus strain 2 (MHV-2) requires

proteolytic cleavage in their target cells, which is mediated by trypsin-like proteases [24, 29, 32]. The situation for group 1 coronavirus is unclear. Recently, the S protein of the group 1 coronavirus HCoV-229E was reported to be cleaved by treatment with cathepsin L and trypsin, which prompts the fusion of the viral envelope and the cell membrane, similar to SARS-CoV [18].

The present study reports the putative role of trypsin in cell-adapted PEDV infection of Vero cells. Trypsin treatment was performed in the early and late stages of viral infection, and its influence on viral titer and syncytium formation was examined. Furthermore, the effect of trypsin on the S protein was compared in free and receptor-bound virions. The results suggest that trypsin activity is involved mainly with receptor-bound S protein of PEDV, leading to the conclusion that the effect of trypsin on PEDV is similar to that of the group 2 coronaviruses, SARS-CoV and MHV.

Materials and methods

Cells, virus and antibodies

African green monkey kidney cells (Vero, CCL-81) were prepared in minimum essential medium (MEM, Gibco) supplemented with 5% fetal bovine serum (FBS, Gibco). The cell-adapted strain of the Korean PEDV isolate, KPEDV-9, was propagated as described elsewhere [15], with some modifications. Briefly, Vero cells were inoculated with KPEDV-9 at a multiplicity of infection ≥ 1 and cultured in serum-free MEM at 37°C, 5% CO_2 for 48-60 h. The supernatant was harvested and then clarified by centrifugation at 12,000 g for 10 min at 4°C. Concentration and partial purification were performed by ultracentrifugation under a 20% sucrose cushion at 26,000 rpm for 3.5 h. The pellet was resuspended in 10 mM phosphate-buffered saline (PBS, pH 7.4) and stored at −70°C.

Mouse polyclonal antibodies against PEDV were generated by immunizing 6-week-old female BALB/c mice (Samtako) intraperitoneally with 1×10^5 focus-forming units (ffu) of purified KPEDV-9 emulsified in an equal volume of complete Freund's adjuvant (Sigma-Aldrich) on day 1 and incomplete Freund's adjuvant (Sigma-Aldrich) on days 14, 21 and 28. Whole blood was collected from the retro-orbital sinus on days 0 and 35 and centrifuged at 1500 g for 10 min to separate the sera.

Virus infection

Cultured Vero cells were inoculated with KPEDV-9 as described above and allowed to adsorb for 2 h at 37°C. The Vero cells were washed twice with PBS and cultured in serum- and trypsin-free MEM or MEM containing trypsin (10 μg/ml, Sigma-Aldrich). At 8, 12, 24, and 48 h post-inoculation (hpi), culture supernatants were collected for titration in a focus-formation assay, and cells were fixed with 4% formaldehyde in PBS for 30 min and permeabilized with 1% NP-40 (Sigma-Aldrich) in PBS, followed by immunocytochemistry using mouse anti-PEDV polyclonal sera [8]. Clusters of infected cells staining dark gray were counted under an inverted microscope and reported as ffu.

Trypsin treatment at various stages of virus infection

Cells or viruses were treated with trypsin at various stages of virus infection as described in Fig. 1. To investigate the effects of proteolytic cleavage of the surface protein of Vero cells and free virions by trypsin, Vero cells or KPEDV-9 were pre-treated with trypsin prior to infection. Trypsin treatment was performed for 30 min at 37°C prior to inoculation, and enzyme activity was neutralized with 2 μg/ml aprotinin (Sigma-Aldrich). Trypsin-pretreated Vero cells were inoculated with KPEDV-9, and untreated Vero cells were inoculated with trypsin-pretreated KPEDV-9. After a 2-h incubation to allow adsorption, cells were washed three times with PBS and then cultured in serum-free MEM without trypsin for 24 h.

In another experiment, trypsin treatment was carried out during the virus adsorption stage to determine whether trypsin is involved in the entry of KPEDV-9. Vero cells were inoculated with KPEDV-9 in the presence of various concentrations of trypsin (5, 10, 20, 40 and 80 μg/ml) during the adsorption period and were cultured in serum-free MEM at 37°C for 24 h. To investigate the effect of trypsin on the budding stage of PEDV infection, KPEDV-9-infected Vero cells were prepared by inoculating them with purified KPEDV-9 and then cultured in MEM for 20 h. Prior to trypsin treatment, the cell monolayer was washed three times with PBS to remove residual FBS and released virions, prior to treatment with trypsin for 10 min. After neutralization of trypsin by the addition of aprotinin, cells were cultured for an additional 4 h. The culture supernatants were harvested for virus titration, and cells were fixed for immunocytochemistry at the indicated times.

Immunocytochemistry

Virus-infected cells were detected by probing with mouse anti-PEDV polyclonal antisera and biotinylated rabbit anti-mouse IgG and visualized by treatment with streptavidin-biotinylated horseradish peroxidase (Vector Labs) followed by 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB, Vector Labs). All specimens were observed under an inverted microscope.

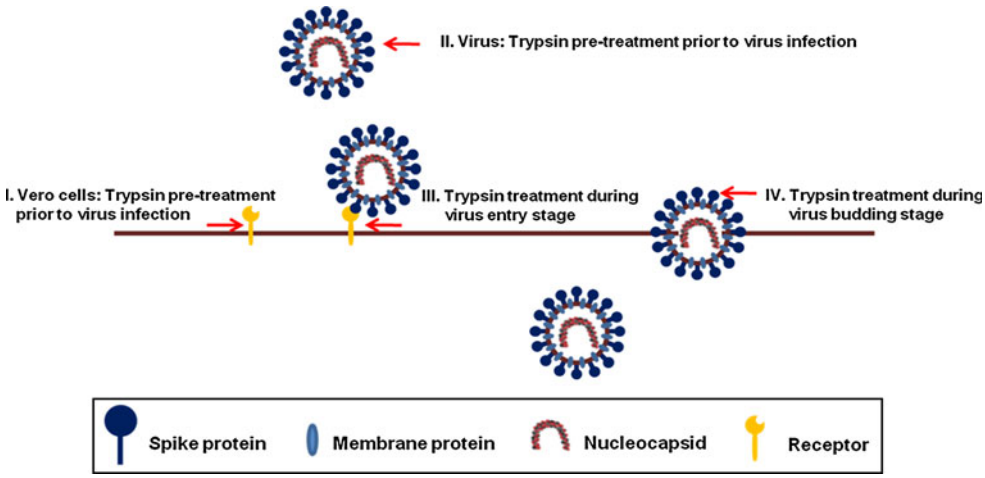

Fig. 1 Diagram of trypsin treatment at various stages of virus infection. To investigate the involvement of trypsin on virus infection, trypsin treatment was done at various stages of virus infection, such as prior to virus inoculation (Vero cells or virus), during the virus entry stage and during the virus budding stage. Trypsin was inactivated by aprotinin. Trypsin action points are indicated by arrows

Treatment of PEDV S protein with trypsin

Ultrapurified KPEDV-9 was treated with various concentrations of trypsin at room temperature (RT) for 10 min and then analyzed by western blotting. Vero cells were infected with KPEDV-9 in the absence of trypsin for 24 h, and KPEDV9-infected Vero cells then were harvested and treated with trypsin for 10 min at RT. Mock-infected trypsin-treated Vero cells were used as a negative control. Samples for western blot analysis were treated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and electrophoresed in an 8% SDS-PAGE gel system. The separated proteins were electrically transferred onto a polyvinyl difluoride membrane (Amersham Bioscience). The antibodies used in this study were mouse anti-PEDV polyclonal antibodies against PEDV S protein, and monoclonal anti-β-actin-peroxidase (Sigma-Aldrich). The bands were visualized using Supersignal West Dura (Pierce) with LAS-1000PLUS (Fujifilm).

Statistical analysis

Statistical analysis was performed using SPSS, version 7.5, for Windows. Correlation coefficients were calculated using Pearson's correlation coefficient. Error bars represent the standard deviations from at least three replicates.

Results

Trypsin is not essential for PEDV infection

PEDV propagation in Vero cells results in low infection rates, even after subsequent passages in the absence of trypsin supplementation [15]. However, with the addition of trypsin, virus adaptation to Vero cells increased, and a prominent cytopathic effect (CPE) marked by formation of syncytia was observed in subsequent passages. Following this observation, the growth rate of KPEDV-9 in Vero cells in the presence or absence of trypsin supplementation was compared. As shown in Fig. 2, detectable levels of progeny virions were observed from 8 hpi in both trypsin- and non-trypsin-supplemented media. At 12 hpi, the titer was higher (3.09×10^3 ffu/ml) in trypsin-supplemented samples than in non-trypsin-supplemented samples (1.08×10^3 ffu/ml). The rate of virus production in trypsin-supplemented cultures was also significantly higher than in trypsin-free cultures (virus titer of 1.83×10^5 and 1.65×10^4 ffu/ml at 24 hpi, respectively). Even at 48 hpi, the titer in the trypsin-free cultures only reached peak titer levels of 4.52×10^4 ffu/ml, which was significantly lower than the peak titer attained at 24 hpi in trypsin-supplemented cultures. These results are consistent with the suggestion that trypsin is not absolutely essential for Vero-cell-adapted PEDV infection, as reported for other group 1 coronaviruses, but the titer increases during infection with trypsin-treated virus.

Trypsin mediates the penetration of cell-attached PEDV

To investigate how trypsin enhances PEDV infectivity of Vero cells, trypsin was added during various stages of infection. Trypsin treatment of KPEDV-9 prior to inoculation did not significantly differ from non-trypsin-treated virus after 20 hpi (1.24×10^4 and 1.32×10^4 ffu/ml, respectively) (Fig. 3). This suggests that proteolytic processing of the surface glycoprotein by trypsin prior to receptor binding does not have a significant effect on enhancing infectivity. To determine whether trypsin interaction with Vero-cell-surface proteins contributes to enhanced PEDV infectivity, Vero cells were pre-treated with 10 μg/ml trypsin for 30 min before inoculation. This treatment did not significantly alter virus titer when compared to the untreated cells. Interestingly, addition of trypsin immediately after inoculation during the absorption

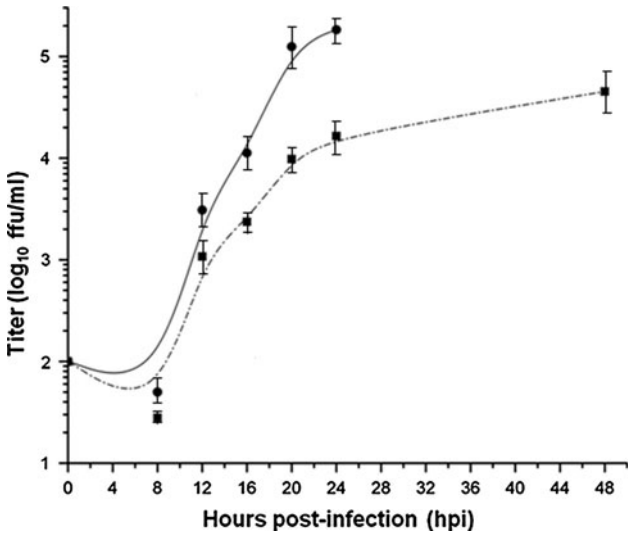

Fig. 2 KPEDV-9 growth curve in Vero cells. The infectivity titer of KPEDV-9 in Vero cells were monitored for 48 hpi in the absence (■) or presence (●) of trypsin. Virus growth in MEM containing 10 μg/ml trypsin resulted in a faster growth rate and higher titers compared to virus cultured in serum-free MEM

period produced about a twofold increase in titer (3.32×10^4 ffu/ml). Trypsin treatment during the period between virus attachment and penetration into Vero cells slightly increased the titer and the number of infected cells.

To investigate the mechanism of trypsin in more detail, Vero-cell-bound PEDV was treated with trypsin. Vero cells were inoculated with KPEDV-9 in serum- and trypsin-free media for 2 h and then washed twice to remove un-bound KPEDV-9. The cell-bound KPEDV-9 was treated with different concentrations of trypsin for 10 min, and the titers of penetrating and produced virus were determined. When the concentration of trypsin in the medium was increased from 5 μg/ml to 80 μg/ml, the number of PEDV penetrating the Vero cells also increased from 4×10^2 ffu/ml to 9×10^2 ffu/ml. The enhanced trypsin-mediated penetration during initial infection resulted in an increase in virus titer at 24 hpi, from 3.0×10^4 ffu/ml to 1.0×10^5 ffu/ml (Fig. 4). These findings are consistent with the notion that trypsin activity during the initial stage of virus infection enhances the efficiency of virus penetration into Vero cells, thereby increasing viral infectivity. Although the penetration of cell-attached virions was facilitated by trypsin treatment, virions treated with trypsin before cell attachment did not show any difference when compared to the results obtained in the absence of trypsin. Based on these findings, it is appropriate to suggest that trypsin might only affect the receptor-bound spike, inducing fusion between the cell membrane and the virus envelope, leading to increased virus penetration.

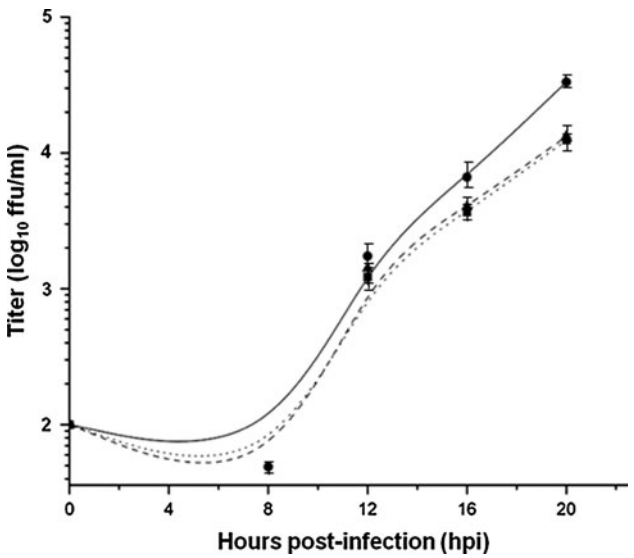

Fig. 3 KPEDV-9 growth curve after trypsin pre-treatment of Vero cells (■) or KPEDV-9 (▲) and trypsin treatment during the virus entry stage (●). Vero cells and KPEDV-9 were pre-treated with trypsin prior to virus inoculation. Trypsin was added during the virus entry stage. Progeny virus was titrated at 20 hpi by FFA. Trypsin treatment of KPEDV-9 immediately after receptor binding resulted in a higher virus titer after 20 hpi post-infection as compared with non-treated and trypsin-pretreated KPEDV-9

Syncytium formation of PEDV-infected Vero cells induced by trypsin treatment

To investigate the role of trypsin on the late stage of infection and syncytium formation, KPEDV-9-infected Vero cells were prepared by inoculation for 20 h. The cells were washed extensively and treated with various concentrations of trypsin for 10 min prior to continuing cell cultivation in fresh serum- and trypsin-free medium. KPEDV-9-infected Vero cells did not show visible signs of syncytium formation in the absence of trypsin, while KPEDV-9-infected Vero cells treated with 5, 10 and 20 μg/ml trypsin at 20 hpi contained multiple syncytia (Fig. 5A). Without trypsin treatment, the virus titer at 4 hpi was 1.4×10^3 ffu/ml while KPEDV-9-infected Vero cells treated with 5, 10, 20, 40 and 80 μg/ml trypsin showed virus titers of 2.1×10^3, 3.0×10^3, 3.5×10^3, 4.1×10^3 and 6.7×10^3 ffu/ml, respectively (Fig. 5B). In virus budding stage, trypsin also activated syncytium formation of infected Vero cells and consequently increased virus infectivity. Newly packaged virions budding from infected Vero cells could be activated by trypsin, which caused the infected Vero cells to form syncytia. This finding was consistent with the previous results shown in Figs. 3 and 4. The collective results supported the idea that trypsin acts on cell-attached virions, both during virus attachment and during virus release and induces membrane fusion between the host-cell membrane and the virus envelope, and also between host-cell membranes.

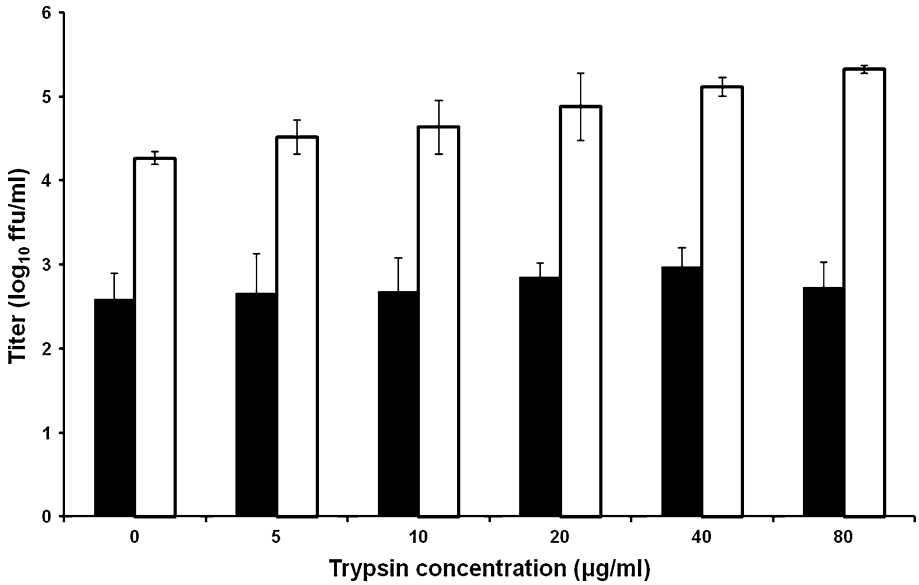

Fig. 4 Enhancement of cell-attached KPEDV-9 penetration by trypsin. Vero cells were inoculated with KPEDV-9 for 2 h and then washed to remove unbound KPEDV-9. Only cell-attached KPEDV-9 was treated with trypsin for 30 min, and penetrated virus (■) and progeny virus were titrated after 24 h (□). Increasing the amount of trypsin added during virus adsorption resulted in increased virus penetration into Vero cells during initial entry and higher virus titers after 24 hpi

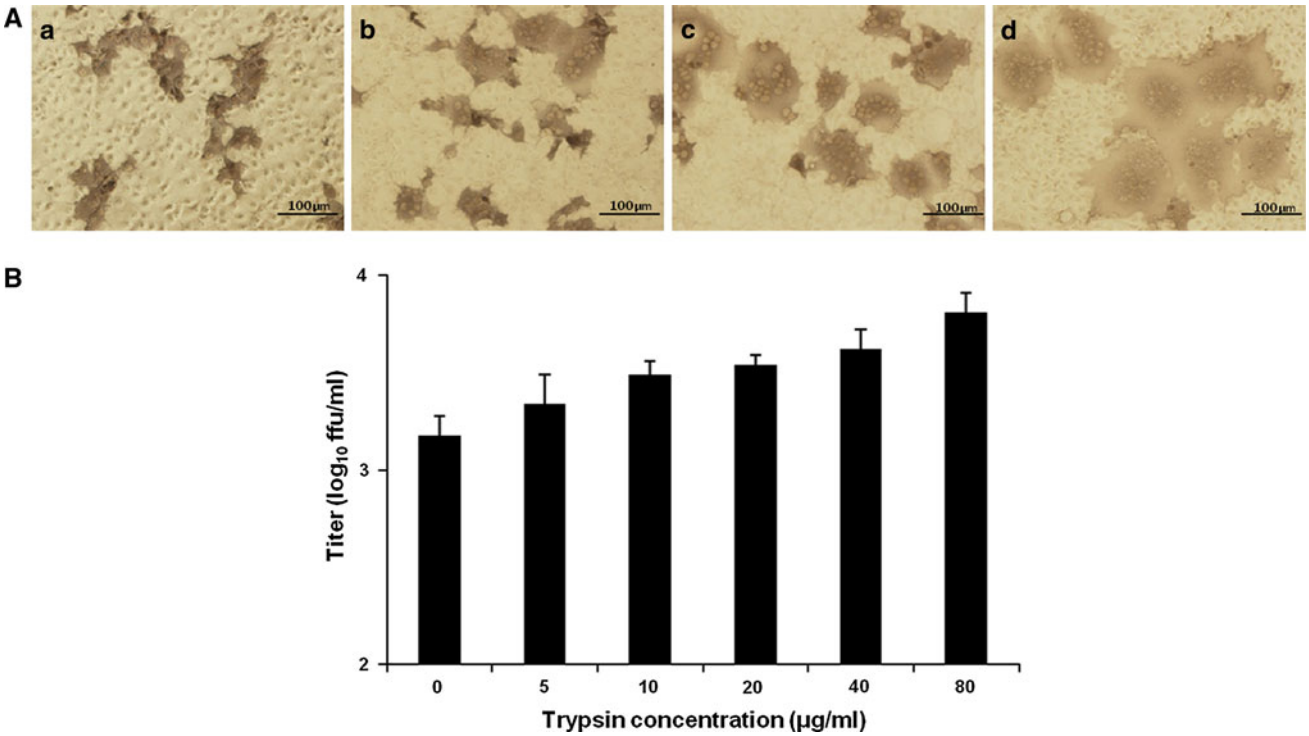

Fig. 5 Syncytium formation by KPEDV-9-infected Vero cells induced by trypsin. Vero cells infected with KPEDV-9 were left untreated or treated with different concentrations of trypsin (a, 0 μg/ml; b, 5 μg/ml; c, 10 μg/ml, and d, 20 μg/ml) for 10 min. Cells were cultured for an additional 4 h and observed for syncytium formation (**A**), and the virus was titrated (**B**)

Cleavage of receptor-bound S protein by trypsin

The S protein from ultrapurified virions and receptor-bound virions was treated with trypsin and analyzed by western blotting. S protein from both ultrapurified virions and KPEDV-9-infected Vero cells that had not been treated with trypsin was apparent as a species of about 220 kDa, which represented the glycosylated native S protein (Fig. 6). In ultrapurified virus, only this protein species was detected, even after trypsin treatment, while 140-kDa and 125-kDa proteins, likely trypsin-cleaved S protein, were detected in trypsin-treated KPEDV-9-infected Vero cells (Fig. 6). The findings supported the suggestion that PEDV S protein has a site that is highly sensitive to trypsin cleavage to produce two fragments, as has been reported for other coronaviruses [23, 24, 32]. However, this

cleavage only occurred when the virus was associated with receptor protein.

Discussion

Several enterotropic or pneumotropic viruses, such as those belonging to the families *Orthomyxoviridae* and *Paramyxoviridae*, undergo proteolytic cleavage of their surface glycoprotein prior to entry into host cells to facilitate virus penetration by activating the fusion domain of the surface glycoprotein [6, 26]. The activated fusion protein undergoes conformational changes that induce fusion of the viral envelope and host membranes [3, 27, 30]. This process usually occurs during the period between virus maturation and virus attachment to the host receptors [25, 33]. After the fusion process, the viral core, including the viral genome, is transported into the cytoplasm where uncoating and replication ensue [22]. In natural infections, the viral surface glycoproteins that are not cleaved during maturation are subsequently cleaved by exogenous proteases secreted from host pancreas, liver and bronchiolar epithelia [19]. In cell culture, the protease cleavage that is required for virus propagation is carried out by exogenous proteases such as trypsin or pancreatin [1, 15, 28]. These exogenous proteases induce syncytium formation by activating the fusion domain of the viral glycoprotein expressed on the surface of infected cells [2, 16].

Several studies of different coronaviruses have shown that proteolytic cleavage of the S protein enhances viral infectivity. For MHV, separation of the S1 and S2 subunits enhances the fusion activity of the S2 subunit and increases viral infectivity [5, 32]. Mutations that alter the furin protease recognition sequence (RXR/KR) located at the junction of the S1 and S2 subunits as well as treatment with a peptide furin inhibitor prevent the proteolytic cleavage of the S protein, resulting in reduced cell-cell fusion activity, although viral entry is not significantly affected [4, 9, 14]. Conversely, the addition of trypsin to the culture medium can enhance the fusion of MHV-infected cells [13]. In contrast to MHV, the S protein of SARS-CoV does not show any evidence of proteolytic maturation to cleaved S1 and S2 subunits in mature virions [35]. Instead, proteolytic cleavage of the S protein on the surface of infected cells occurs by exogenous proteases, mediating cell-cell fusion [25, 29].

Similar to SARS-CoV, the S protein in most group 1 coronaviruses also does not exhibit cleaved S1 and S2 subunits during virus maturation and biogenesis [34]. Several studies on TGEV and PEDV have used trypsin-supplemented culture media to induce CPE by syncytium formation in ST cells and Vero cells, respectively [15, 31]. Furthermore, in the case of PEDV, trypsin facilitates successful propagation in Vero cells as well as other primate cell lines [15, 20]. However, the role of cellular and exogenous proteases on the cell entry of group 1 coronaviruses, particularly PEDV, as well as the putative proteolytic cleavage site on the S protein, remains unclear. Based on the present results summarized in Figs. 3, 4 and 5, enhancement of virus penetration and cell-cell fusion induced by the addition of trypsin suggests that the PEDV S protein may also be cleaved into S1 and S2 subunits during the course of infection. Electrophoretic examination of purified virions resulted in the detection of the PEDV S protein as a monomer of about 220 kDa in the absence of trypsin, while the protein was detected as two fragments of 140 kDa and 125 kDa in the presence of trypsin (Fig. 6). It may be that the PEDV S protein on native virions adopts a conformation that protects it from various exogenous proteases, but the S protein attached to the host receptor protein may undergo a conformational change that exposes a trypsin cleavage site. Previous reports have described the formation of syncytia by PEDV-infected cells only upon addition of trypsin in the culture medium, and sequence analysis of the PEDV S protein has revealed the absence of the RRX(R/H)R motif, which is associated with cleavage into the S1 and S2 subunits [11]. This suggests that the PEDV surface glycoprotein does not undergo proteolytic processing upon maturation and release. Conversely, the observation that trypsin can induce

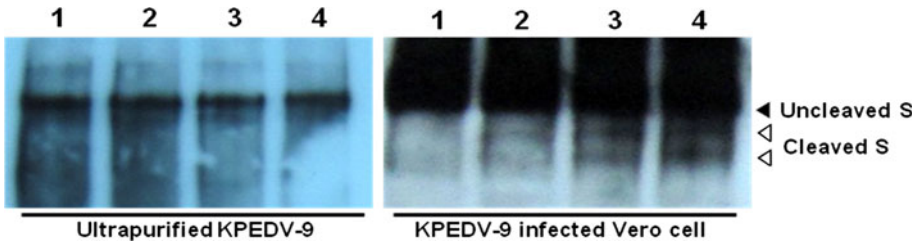

Fig. 6 Western blot analysis of the PEDV S protein after treatment with trypsin. Ultrapurified KPEDV-9 virions (left) and KPEDV-9-infected Vero cells (right) were treated with trypsin (lane 1, 0 μg/ml; lane 2, 10 μg/ml; lane 3, 50 μg/ml, and lane 4, 100 μg/ml) at room temperature for 10 min. PEDV S was detected using anti-PEDV polyclonal antibodies raised in mice. Uncleaved S protein and cleaved S protein are indicated by black and white arrows, respectively

cell-cell fusion in PEDV-infected cells suggests that proteolytic processing of the S protein by exogenous trypsin may augment viral entry by facilitating fusion of the viral membrane with the host membranes [11, 15].

It seems that the timing of the cleavage of the S protein by trypsin is critical for the activation of fusion activity. As shown in Fig. 3, early activation of the S protein before binding to cellular receptors did not enhance viral entry into the host cell, while addition of trypsin shortly after receptor binding increased the efficiency of virus entry. While the S protein in infected cell lysates and receptor-bound virions was cleaved into two fragments, the S protein cleavage in PEDV was different from that of other coronaviruses, as PEDV S protein was only cleaved when associated with its host cell. This implies that cleavage of the S protein by trypsin occurs only when it is bound at the surface of host cells to the host receptor protein, which presumably induces a conformational change in the bound S protein. This conformational change might expose a trypsin cleavage site. Cleavage of the S protein could result in membrane fusion. It would be of interest to determine the nature of the conformational change that is involved and the location of the S protein cleavage site.

In summary, the present results reveal the role and importance of trypsin in PEDV infection of Vero cells. Trypsin is not essential for PEDV infection but enhances its infectivity and CPE formation. Trypsin cleaves PEDV S protein only when it bound its cell receptor and in the later stages of infection. The association of the S protein with the host receptor could induce conformational changes that expose a trypsin cleavage site(s). The resulting cleavage might expose or activate the fusion peptide and activate PEDV entry and pathogenesis.

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