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Title: **A single point mutation creating a furin cleavage site in the spike protein renders porcine epidemic diarrhea coronavirus trypsin-independent for cell entry and fusion.**

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Running title (max.54 characters incl. spaces): PEDV S protein activation by furin protease

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Abstract short (75 words)

The emerging porcine epidemic diarrhea virus (PEDV) requires trypsin supplementation to activate its S protein for membrane fusion and virus propagation in cell culture. By substitution of a single amino acid in the S protein we created a recombinant PEDV (PEDV-S^{FCS}) with an artificial furin protease cleavage site N-terminal of the putative fusion peptide. PEDV-S^{FCS} exhibited trypsin-independent cell-cell fusion and was able to replicate in culture cells independent of trypsin, though to low titer.

Text:

Entry of the enveloped coronaviruses (CoV) is mediated by the spike (S) glycoprotein. The S protein can be functionally divided into two domains, S1 and S2, which enable the consecutive stages of receptor binding and membrane fusion, respectively. Like other class I viral fusion proteins, the CoV S proteins rely on proteolytic cleavage by host proteases for fusion priming/activation; cleavage enables the release of the fusion peptide and its insertion into the target cell membrane in a controlled manner. To this end most CoV exploit endogenous cellular proteases such as furin, plasma membrane proteases and endolysosomal proteases that cleave the S protein during virus exit from the infected cell or during its entry at or in the target cell (1, 2). Cleavage of CoV S proteins has been shown to occur at the junction of the receptor binding domain S1 and the membrane fusion domain S2, and just upstream of the putative fusion peptide (S2' site) in the S2 domain, with the latter cleavage supposed to be the most critical for fusion as it liberates the fusion peptide (1, 2).

Porcine epidemic diarrhea virus (PEDV) is an emerging coronavirus that causes acute diarrheal disease in swine in Asia and since 2013 in the Americas. In contrast to other CoV, PEDV requires the presence of an exogenous protease, trypsin, in the cell culture medium to activate the S protein for membrane fusion and for virus propagation in cell culture (3). This trypsin dependency *in vitro* is consistent with the tissue tropism of PEDV *in vivo* where PEDV is confined to the trypsin-rich small intestine. Trypsin activates the PEDV S protein for membrane fusion after the binding of the virion to the host cell (4). Genetic and mutational analyses have pointed to a conserved arginine just upstream of the fusion peptide (S2' site) in the membrane fusion domain S2 as the trypsin cleavage site for fusion activation, yet direct evidence of functional cleavage at this position is lacking (4).

To assess the cleavage position required for PEDV S protein fusion activation and to test whether activation by exogenous trypsin can be bypassed by an endogenous host protease, we introduced an artificial cleavage recognition site for the furin protease into the PEDV spike protein (Fig.1). The proprotein convertase furin is a type I membrane protein that is ubiquitously expressed in eukaryotic cells. Furin is primarily located in the *trans*-Golgi network but also occurs at the cell surface and as an extracellular, truncated soluble form (5). It cleaves cellular and viral proteins C-terminal of a substrate recognition motif containing basic amino acids with R-X-X-R and R-X-K/R-R representing the minimal and highly favored motif, respectively (R, arginine; K, lysine; X, any amino acid). We constructed a mutant PEDV S protein (strain CV777) with a valine to arginine single residue substitution at amino acid position 888 (V888R) thereby creating a furin cleavage sequence site (*V*QKR →*R*QKR) N-terminal of the predicted fusion peptide (6). Bioinformatic analysis

predicted that this position can be cleaved by furin with a prediction score of 0.875, relative to 0.611 for the wildtype S protein (Fig.1a).

First, we assessed whether the introduction of the furin cleavage site (FCS) in the S protein enables trypsin-independent cell-cell fusion. Therefore, we transiently expressed the S^{FCS} protein and the S^{WT} protein, each provided with a C-terminally appended Flag-tag, in Vero cells. At 6 h post transfection, the culture medium was replaced by fresh medium supplemented either with furin inhibitor or with soybean trypsin inhibitor, the latter to ensure that trypsin activity was completely absent. At 47 h post transfection, cells were treated with trypsin for 1 h or left untreated. In the presence of trypsin, the S^{WT} and S^{FCS} spike proteins were both able to efficiently form syncytia (Fig.1b). As expected, the S^{WT} spike protein was unable to mediate cell-cell fusion when trypsin was omitted from the cell culture medium. However, formation of multiple, large syncytia was seen after expression of the S^{FCS} spike protein in the absence of trypsin activity. Trypsin-independent syncytia formation by S^{FCS} could be inhibited by the inclusion of furin inhibitor in the culture medium (Fig.1b). These data indicate that the creation, through a single amino acid substitution, of a furin cleavage site at the S2' position renders the PEDV S protein prone to activation of its membrane fusion capacity by endogenous furin.

To assess whether the furin cleavage site at the S2' position of the S protein enables the virus to infect cultured cells independent of trypsin, we introduced the mutation encoding the S^{V888R} substitution into the viral genome. Recombinant viruses were generated encoding either the wildtype CV777 S protein (PEDV-S^{WT}) or the S^{FCS} mutant (PEDV-S^{FCS}) using our recently established PEDV reverse genetics system (7). To facilitate the analyses, the non-essential ORF3 gene in the genome of both recombinant viruses was replaced by the GFP gene. Recombinant PEDV-S^{WT}

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and PEDV-S^{FCS} viruses were successfully rescued in the presence of trypsin and the relevant S gene region of their genome was confirmed by sequencing. Next, we inoculated Vero cells in parallel with PEDV-S^{WT} and PEDV-S^{FCS} in the absence and presence of trypsin for 2 h. The infection was subsequently continued in the absence or presence of trypsin for another 10 h after which infected (i.c. GFP-positive) cells were visualized by fluorescence microscopy. In the absence of trypsin, hardly any infection was seen on Vero cells for the PEDV-S^{WT} virus whereas clear infection was observed for the PEDV-S^{FCS} mutant (Fig.2a). Both viruses efficiently infected Vero cells in the presence of trypsin. Syncytia were abundantly observed when trypsin was absent after virus inoculation for the PEDV-S^{FCS} mutant, but not for the PEDV-S^{WT} (Fig.2a and 2b). These results confirm our earlier observation that the furin recognition site creating mutation S^{V888R} confers trypsin-independent cell-cell fusion.

To assess whether the S^{V888R} mutation would also enable PEDV to propagate in the absence of trypsin, the growth kinetics of the PEDV-S^{WT} and PEDV-S^{FCS} viruses on Vero cells were compared in the absence and presence of trypsin. Vero cells were inoculated in parallel with PEDV-S^{WT} and PEDV-S^{FCS} at an MOI of 0.01 or 0.1. Both viruses displayed similar growth kinetics and reached similar titers in the presence of trypsin (Fig.2c). In the absence of trypsin, no infectious progeny was detected with either virus after inoculation at low MOI (MOI 0.01, data not shown). At 10-fold higher MOI the PEDV-S^{FCS} – but not the PEDV-S^{WT} – yielded low infectious virus titers starting from 12 h post infection. To check the cleavage status of the spike protein as it occurs on PEDV-S^{FCS} virions we performed western blot analysis. The results indicate that the introduction of the furin cleavage site at the S2' site did not result in a detectable cleavage (Fig.2e).

Huh-7 human hepatoma cells are known to express high levels of furin protease (8). We therefore analysed whether these cells could support infection by the PEDV-S^{FCS} virus. Of note, infection of Huh-7 cells in the presence of trypsin could not be assessed since these cells are affected too strongly by trypsin at the concentrations required for propagation of PEDV in cell culture. Inoculation of Huh-7 cells with PEDV-S^{FCS} virus resulted in trypsin-independent infection and development of syncytia (Fig.3a and b). Virus entry as well as cell-cell fusion could be inhibited when furin inhibitor was present during these processes. Interestingly, some PEDV-S^{WT} infection was also observed on Huh-7 cells which could be inhibited by furin inhibitor, yet no syncytia were seen. Whether this infectivity correlates with the predicted suboptimal proprotease cleavage site at the S2' position in the PEDV S^{WT} protein (Fig.1) remains to be seen.

The proteolytic activation process of the CoV spike fusion protein has long been rather enigmatic. Whereas processing at the S1/S2 junction by furin was documented already in the early 80s (9, 10), this cleavage occurs only in a subset of coronaviruses and does not liberate the putative fusion peptide at the N terminus of the membrane-anchored subunit, as it does in other class I viral fusion proteins. The second, more universal as well as more appropriately located S2' cleavage site was identified only recently and evidence for its general importance in CoV infection has since been accumulating ((4, 11-13); for a recent review, see (1)). Cleavage at the S2' position is generally carried out by cellular proteases occurring at the plasma membrane or in the endo-/lysosomal system, depending on the particular target cell and S2' sequence. In the case of PEDV, however, of which the S protein lacks a canonical furin cleavage site at the S1/S2 and the S2' position, activation supposedly occurs by trypsin-like enzymes in the gut and the virus hence requires

supplementation of trypsin for propagation *in vitro*. Earlier we mapped this trypsin-requirement to the S2' cleavage site in the PEDV S protein and demonstrated the critical importance of the characteristic arginine at this site for the viability of the virus and for the cell fusion capacity of the S protein (4).

In the present study we aimed to demonstrate the requirement for cleavage at the S2' site and to alleviate the trypsin dependence of PEDV infection *in vitro*. Thus, we show that introduction - through a single point mutation - of an artificial furin cleavage motif N-terminal of the spike fusion peptide confers cell-cell fusion and PEDV entry in a trypsin independent manner. Both processes were blocked by a furin-specific inhibitor, thereby confirming the functionality of furin cleavage at the S2' position. The observations add further evidence that cleavage just upstream of the fusion peptide is a general and essential requirement for activation of CoV spike proteins for membrane fusion (11-14). Propagation of the PEDV-S^{FCS} in the absence of trypsin was less efficient than in its presence, which might be due to trypsin also being required for virus release (15). Moreover, besides cleavage N-terminal of the fusion peptide, additional cleavage(s) may be required to increase the S protein's membrane fusion efficiency. Cleavage of the S protein at the S1/S2 junction of the coronaviruses MERS-CoV, SARS-CoV and IBV has been implicated to precede and promote cleavage at the S2' position (11, 13, 14). For PEDV, the lack of efficient cleavage of virion-incorporated S^{FCS} proteins indicates that the introduced furin cleavage site at the S2' site is rather inaccessible for furin. Whether cleavage at the S1/S2 junction or binding to the receptor enhances the efficiency of cleavage at the S2' cleavage site awaits further investigation.

While field strains of PEDV strictly require exogenous trypsin for propagation *in vitro*, serial passaging of the virus on cultured cells can lead to trypsin

independency (3, 4). Vero cells are the most commonly used cells for PEDV studies because of their resistance to the high concentrations of trypsin required for PEDV infection. The protease requirement of PEDV may hence – in addition to the specific virus receptor – function as a critical tropism determinant *in vitro* as well as *in vivo*. As a consequence, the rational or evolutionary adaptation of CoV to the use of ubiquitous, endogenous proteases like furin for the activation of their S proteins may expand their tropism *in vitro* and *in vivo* (1).

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Figure legends:

Fig.1: The PEDV S protein with an artificial furin cleavage site at the S2' position (S^{FCS}) mediates trypsin-independent cell-cell fusion. (a) Schematic representation of the PEDV S protein (drawn to scale). Indicated are the position of the receptor binding domain S1 and the membrane fusion domain S2, predicted *N*-glycosylation sites (Ψ; NetNGlyc server), predicted S2' cleavage site (red triangle), fusion peptide (orange bar), and transmembrane domain (black bar; TMHMM server). Lower panel: Amino acid sequence at the putative S2' cleavage site (R891, indicated in bold) for the wildtype CV777 S protein (S^{WT}) and for a mutant S protein (S^{FCS}) with a valine to arginine substitution at amino acid position 888 (colored in red) creating a furin cleavage site sequence (underlined: R-X-(K/R)-R; X: any amino acid). Furin scores for the S2' position predicted by the ProP 1.0 server are indicated for both S variants. (b) Vero cells were transfected with expression plasmids encoding wildtype PEDV S (S^{WT}) or S^{FCS} and cultured in the absence or presence of furin-inhibitor (FI; 40 μM), soybean trypsin inhibitor (SBTI; 40 μg/ml) or trypsin (15 μg/ml) as indicated. At 40 h post transfection cells were fixed and permeabilized. Nuclei (blue) were stained with DAPI and PEDV S (green) expression was visualized by its C-terminally appended FLAG-tag using anti-Flag MAb (Sigma). The transfection experiments were repeated two times, representative images are shown.

Fig.2: Recombinant PEDV virus encoding the S of PEDV-CV777 strain with an artificial furin cleavage site at the S2' site (PEDV-S^{FCS}) mediates trypsin-independent cell-cell fusion. (a) Cells were infected with GFP-expressing recombinant PEDV viruses carrying wildtype CV777 S (PEDV-S^{WT}) or mutated CV777 S (PEDV-S^{FCS}) in the presence of trypsin (15 μg/ml) or soybean trypsin inhibitor (SBTI; 40 μg/ml) as

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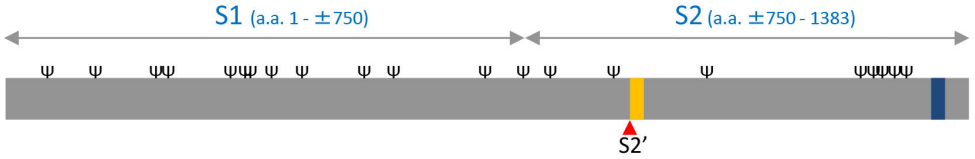
indicated. At 2 h post infection cells were washed and further cultured in the presence of soybean trypsin inhibitor (SBTI; 40 µg/ml) or trypsin (15 µg/ml) as indicated. Cells were fixed at 12 h p.i. Nuclei were stained with DAPI (blue) and images of PEDV-infected cells (GFP-positive, green) were acquired. Experiments were repeated two times and representative images are shown. (b) Vero cells were infected with PEDV-S^{WT} and PEDV-S^{FCS} in the presence of trypsin for 2 h and further cultured in the presence of soybean trypsin inhibitor (as indicated above). At 12 h p.i. the numbers of nuclei per focus of infection were counted. (c) Vero cells were infected with recombinant PEDV-S^{WT} and the PEDV-S^{V888R} (MOI 0.01) in the absence or presence of trypsin. The infectivity in the culture medium was monitored by taking small samples from the medium at various time points post infection and titration on Vero cells in the presence of trypsin. (d) Vero cells were mock infected or infected with PEDV-S^{WT} and PEDV-S^{FCS} in the presence of trypsin inhibitor for 2 h (MOI 10) and further cultured in the absence of trypsin, as described under (a). At 24 h p.i. cell culture supernatant of these cultures was pelleted through a 20% sucrose cushion. Pellets were dissolved in Laemmli sample buffer and subjected to western blotting. Virion-incorporated S^{WT} and S^{FCS} proteins were detected by their C-terminally appended FLAG-tag using an anti-Flag MAb (Sigma). Sizes of marker proteins are indicated in kilodaltons.

Fig.3: Furin inhibitor blocks trypsin-independent entry and formation of syncytia by PEDV-S^{FCS} in human hepatoma (Huh-7) cells. (a) Cells were inoculated with PEDV-S^{WT} or PEDV-S^{FCS} at MOI 0.5 (MOI based on virus infectivity on Vero cells in the presence of trypsin). Furin inhibitor dec-RVKR-CMK (FI, 40 µM) was present in the cell culture supernatant from -1h to 2 h p.i. or from 2 h to 12 h p.i.. To prevent trypsin

mediated entry, soybean trypsin inhibitor (SBTI; 40 µg/ml) was present in the culture supernatant throughout the experiment. Cells were fixed at 12 h p.i.. Nuclei were stained with DAPI (blue) and images of PEDV-infected cells (GFP-positive; green) were acquired. Experiments were repeated two times and representative images are shown. (b) Huh-7 cells were infected with PEDV-S^{WT} and PEDV-S^{FCS} in the presence of trypsin inhibitor for 2 h and further cultured in the absence or presence of furin inhibitor, as described under (a). At 12 h p.i. the numbers of nuclei per focus of infection were counted.

a.



	Spike sequence (a.a.885-996)	ProP pred.
S^{WT}	GRVVQK**R**SVIED	0.611
S^{FCS}	GRVRQK**R**SVIED	0.875

b.