Identification of the functional domain of the porcine epidemic diarrhoea virus receptor

Zhifu Shan,† Jiyuan Yin,† Zongying Wang, Peipei Chen, Yijing Li and Lijie Tang

Department of Preventive Veterinary Medicine, College of Veterinary Medicine, Northeast Agricultural University, No. 59 Mucai Street, Harbin 150030, PR China

Porcine aminopeptidase N (pAPN) is a functional receptor for porcine epidemic diarrhoea virus (PEDV). Although PEDV is known to use the pAPN as the major receptor for cell entry, the crucial domain of the pAPN that interacts with the PEDV is still unknown. In the present study, in order to determine the crucial domain of the pAPN, the extracellular domain of pAPN was divided into three subdomains named SPA, SPB and SPC, based on its secondary structure. Recombinant plasmid pcDNA3.1 expressing SPA, SPB and SPC was constructed and introduced into Madin–Darby canine kidney (MDCK) cells by transfection. Following the detection of PEDV infection in transfected MDCK cells after PEDV challenge, we clearly demonstrated that the SPC subdomain plays a key role in cell entry of PEDV and its expression permits PEDV growth in transfected MDCK cells, while virus propagation can be inhibited by anti-SPC serum, indicating that the SPC subdomain appears to be a crucial functional domain in contributing to efficient PEDV infection.

Porcine epidemic diarrhoea virus (PEDV) belonging to the group I coronavirus is the major cause of lethal diarrhoea disease in piglets (Oh et al., 2003; Weiss & Navas-Martin, 2005). Like transmissible gastroenteritis virus, PEDV uses porcine aminopeptidase N (pAPN) as the major receptor for cell entry (Chen et al., 2012; Delmas et al., 1992, 1993; Hansen et al., 1998; Li et al., 2007; Reguera et al., 2012; Schultz et al., 1995; van der Hoek et al., 2004). Remarkably, it was found that the two viruses utilized distinct cultured cell lines for virus propagation in vitro (Delmas et al., 1992; Hofmann & Wyler, 1988). pAPN, characterized as a member of a membrane-bound metallo-protease family, is expressed predominantly on the epithelial cell surface of the kidney, small intestine and respiratory tract (Kenny & Maroux, 1982; Lendeckel et al., 2000; Nam & Lee, 2010). Although pAPN is recognized as the major cell receptor for PEDV entry and initiates cell infection through interaction between the spike protein of PEDV and pAPN (Bosch et al., 2003; Godet et al., 1994), the crucial domain of pAPN that interacts with PEDV is still unknown.

In this study, in order to determine the crucial domain of pAPN that interacts with PEDV, we obtained the protein structure set of pAPN from the SWISS-MODEL web server as described previously (Arnold et al., 2006; Benkert et al., 2011; Biasini et al., 2014), and its transmembrane domains, secondary structure and functional domains were analysed using TMHMM 2.0, the PSIPred-MEMSAT 3 web server and the Predict Protein server, respectively. As shown in Fig. 1(a), the pAPN encompassed an intracellular domain comprising residues 1–11 and an extracellular domain, in which the residues 12–35 forming an α-helix were considered as the transmembrane domain of pAPN. The extracellular domain of pAPN was divided into three subdomains named SPA, SPB and SPC, respectively. SPA (residues 72–369) contained a 15-stranded β-barrel fold, showing structural evidence for the common ancestry of M1 metalloenzymes based on a common structural fold in which zinc activates a water molecule. SPB (residues 370–615) encompassed seven α-helical superhelicis (residues 370–542) and one β-sandwich fold with 7 strands(residues 543–615). SPC (residues 616–963) was composed of 16 α-helices organized as eight HEAT-like repeats, forming a concave face oriented towards the active site of the peptidase.

Subsequently, the gene fragments encoding the SPA, SPB and SPC subdomains were amplified by PCR assay using a full-length pAPN gene as template, and then were respectively subcloned into eukaryotic expression vector pcDNA3.1 (Invitrogen), giving rise to recombinant plasmids

†These authors contributed equally to this work.

The atomic coordinates and structure factors of porcine aminopeptidase N have been deposited in the Protein Data Bank (http://www.rcsb.org/) with ID code 4HOM.
pcDNA3.1-SPA, pcDNA3.1-SPB and pcDNA3.1-SPC in conjunction with a signal peptide gene SP0 for each. The recombinant plasmids were then introduced individually into Madin–Darby canine kidney (MDCK, ATCC CCL-34) cells by transfection with 1.6 µg DNA.

In order to determine the target protein expression in MDCK cells, an immunofluorescence assay for transient transfection was performed. In brief, the transfected MDCK cells were blocked with PBS containing 1% BSA, followed by incubation with mouse anti-pAPN serum (1 : 100) used as primary antibody. The bound antibodies were then detected using FITC-conjugated goat anti-mouse IgG (Beijing-Zhong Shan Golden Bridge) diluted at 1 : 500, and observed using a fluorescence microscope. The results showed that fluorescence can be clearly observed in transfected MDCK cells with pcDNA3.1-SPA, pcDNA3.1-SPB and pcDNA3.1-SPC, respectively (Fig. 1b), but not in MDCK cells transfected with pcDNA3.1, indicating that SPA, SPB and SPC were successfully expressed in MDCK cells, which can be accurately recognized by anti-pAPN antibody.

The transfected MDCK cells were then infected by PEDV (0.1 ml containing 10^{4.5} TCID_{50}) according to a previously described method (Li et al., 2007) 48 h post-transfection. We found that cytopathic effects (CPEs) could be observed microscopically in transfected MDCK cells expressing SPC 72 h after virus challenge, but not in MDCK cells transfected with pcDNA3.1-SPA or pcDNA3.1-SPB (data not shown). In order to detect the presence of PEDV in transfected MDCK cells, total RNA of cell cultures was extracted by kit assay (Fastgene) according to the manufacturer’s instructions, followed by detection using an reverse transcription - PCR (RT-PCR) assay with the primers:

![Fig. 1.](http://vir.sgmjournals.org)
5'-CAGTACCTGTACCGGTGACTTTGT-3' (forward) and 5'-GCCGTGGACTTT-3' (reverse). The results showed that the specific PCR amplicon of PEDV was detected in SPC-expressing MDCK cells, but not in MDCK cells transfected with pcDNA3.1-SPA, pcDNA3.1-SPB or pcDNA3.1 (Fig. 1c). In parallel, an immunofluorescence assay was also performed to detect the presence of PEDV. Briefly, the transfected cell monolayer was fixed with 4% paraformaldehyde at 4°C for 15 min, then permeabilized using 0.2% Triton X-100. After blocking with PBS/1% BSA and incubating with rabbit anti-PEDV polyclonal antibodies (1:100) prepared by Li et al. (2007), the cells were treated with FITC-conjugated goat anti-rabbit IgG (1:200) followed by observation using a fluorescence microscope. As shown in Fig. 1(d), significant fluorescence were observed in SPC-expressing MDCK cells infected with PEDV, while not in MDCK cells expressing SPA or SPB or in pAPN-deficient MDCK cells. Our data indicated that the MDCK cells that normally cannot be used to propagate PEDV permitted PEDV growth due to the presence of SPC, suggesting that the SPC was a functional PEDV-binding domain for cell entry.

Generally, host infection caused by the invasion of virus initiates the interaction between the virus and the host cell receptor. Thus, a specific receptor(s) located on the host cell surface is recognized as the major factor for determining the virus tissue tropism and facilitating virus entry. The cell entry of coronavirus involves two steps: the spike of coronavirus specifically recognizes pAPN surface polysaccharide, followed by an irreversible combination with a cell receptor-binding domain. In this study, the SPC subdomain was determined as the functional subdomain of pAPN for PEDV entry. We investigated the ability of anti-SPC antibody to inhibit PEDV replication in SPC-expressing MDCK cells. The cells were incubated with anti-SPC serum (1:500) prepared by Shan et al. (2012) at 37°C for 30 min, followed by infection with PEDV, and then an immunofluorescence assay was performed 72 h post-infection. The results showed that the anti-SPC antibody could effectively prevent PEDV from infecting SPC-expressing MDCK cells (Fig. 2a). At the same time, the ability of anti-SPC antibody to inhibit PEDV replication in pAPN-expressing MDCK cells was also determined. As shown in Fig. 2(b), the anti-SPC antibody was also able to efficiently block pAPN-expressing MDCK cell infection caused by PEDV similar to the anti-pAPN antibody described previously (Delmas et al., 1992; Liu et al., 2009), while anti-SPA and anti-SPB sera could not. Our data indicated that the anti-SPC antibody could effectively block the interaction between PEDV and the functional domain of pAPN, resulting in virus propagation failure in pAPN-expressing MDCK cells.

In order to evaluate the stability of PEDV replication in SPC-expressing MDCK cells, PEDV was continuously propagated in MDCK cells expressing SPC for five generations. In parallel, pAPN-expressing MDCK cells, Vero cells and MDCK cells treated with liposome were used as control. Following total RNA extraction from each generation of cell cultures, PEDV was detected by RT-PCR assay. The results showed that PEDV was detectable in each generation of SPC-transfected MDCK cell cultures (Fig. 3a), the same as in pAPN-transfected MDCK cells, indicating a good stability for virus replication. Subsequently, virus titres in SPC-expressing MDCK cell cultures and pAPN-expressing MDCK cell cultures, respectively, were determined by testing TCID50. The results showed that there were no significant differences (P>0.05) in levels of virus replication among the SPC-expressing MDCK cell group and Vero cell group treated with liposome (Fig. 3b), while there were no CPEs observed in MDCK cells treated with liposome.

Currently, effective protection against PEDV infection mainly relies on a combination of PEDV-neutralizing antibody and PEDV neutralization epitope (Meng et al., 2014; Ren et al., 2011). However, new mutant virus strains generated under selection pressure could successfully escape neutralizing-antibody-mediated immunity without affecting host infection. Therefore, applying the anti-SPC antibody to prevent PEDV infection will provide a future avenue for protection against PEDV infection.
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References


