

Short Communication

Identification of a putative cellular receptor 150 kDa polypeptide for porcine epidemic diarrhea virus in porcine enterocytes

Jin Sik Oh, Dae Sub Song and Bong Kyun Park*

Department of Microbiology, Virology Lab, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea

Porcine epidemic diarrhea virus (PEDV) causes an acute enteritis in pigs of all ages, often fatality for neonates. PEDV occupies an intermediate position between two well characterized members of the coronavirus group I, human coronavirus (HCoV-229E) and transmissible gastroenteritis virus (TGEV) which uses aminopeptidase N (APN), a 150 kDa protein, as their receptors. However, the receptor of the PEDV has not been identified yet. A virus overlay protein binding assay (VOPBA) was used to identify PEDV binding protein in permissive cells. The binding ability of PEDV to porcine APN (pAPN) and the effects of pAPN on infectivity of PEDV in Vero cells were also investigated. VOPBA identified a 150 kDa protein, as a putative PEDV receptor in enterocytes and swine testicle (ST) cells. Further the PEDV binding to pAPN was blocked by anti-pAPN and pAPN enhanced PEDV infectivity in Vero cells. In conclusion, these results suggested that pAPN may act as a receptor of PEDV.

Key words: PEDV, cellular receptor, porcine aminopeptidase N

Porcine epidemic diarrhea virus (PEDV), a member of the family *Coronaviridae* is an enveloped and single-stranded RNA virus [10]. It causes severe diarrhea in pigs, especially in newborn pigs. PEDV and transmissible gastroenteritis virus (TGEV) are not serologically related to each other, though both infect digestive tract and induce very similar clinical signs [5].

Several members of coronavirus serogroup I, including human coronavirus (HCoV-229E), TGEV, and feline infectious peritonitis virus (FIPV), use aminopeptidase N (APN) as their cellular receptor [6,7]. The highest APN activity is detected in the brush border membrane of the enterocytes and the renal proximal tubule cells [6].

Previously the sequencing of the M, sM, ORF3 and N gene of CV777 strain of PEDV was completed. The S, M, sM, ORF3 and N genes of a British strain of PEDV also been sequenced [3,8,9]. These data indicated that PEDV occupies an intermediate position between two well characterized members of the group I coronavirus, HCoV-229E and TGEV. Both HCoV-229E and TGEV use APN as their receptor [7,11].

A virus overlay protein binding assay (VOPBA) was used for identifying the putative cellular receptor in several viruses [14]. And Schenten *et al.* reported that the soluble form receptor could enhance the infection of HIV (human immunodeficiency virus) [26].

The objectives of this study were to identify a cellular receptor in permissive cells using VOPBA and to determine whether the PEDV infectivity would be enhanced by soluble porcine APN treatment on Vero cells.

The continuous Vero cell line (ATCC, CCL-81) was regularly maintained in α -MEM (minimal essential medium) supplemented with 5% fetal bovine serum (FBS), and 2% antibiotic-antimycotic agent mixture (penicillin, 10,000 IU/ml; streptomycin, 10,000 μ g/ml; and amphotericin B, 25 μ g/ml; Invitrogen, Grand Island, N.Y.). PEDV strain KPEDV-9 which was used for this study has been endorsed to the Green Cross Veterinary Product Co., Ltd. (Suwon, Korea) for manufacturing PEDV live vaccine by the National Veterinary Research and Quarantine Service (Anyang, Korea). KPEDV-9 was propagated in Vero cells with virus replication medium (VM), α -MEM supplemented 0.02% yeast extract, 0.3% tryptose phosphate broth and 2 μ g of trypsin (T-VM), as described previously [22]. And KPEDV-9 was propagated in Vero cells with VM containing pAPN (A-VM) instead of trypsin. ST (swine testicle) and PK-15 (porcine kidney) cells were grown in MEM supplemented with 5% FBS, and 2% antibiotic-antimycotic agent mixture. TGEV, Pyungtak 45 strain was cultured in ST cells.

The monoclonal antibodies for virus overlay protein binding assay (VOPBA) of TGEV and PEDV (S protein) were provided by the National Veterinary Research and

*Corresponding author

Phone: +82-2-880-1255; Fax: +82-2-885-0263

E-mail: parkx026@snu.ac.kr

Quarantine Service, Anyang, Korea. Polyclonal antibody to PEDV was prepared in rabbits using KPEDV-9 strain [22]. Anti-pAPN monoclonal antibody was kindly provided from Dr. Delmas (INRA, France). For the assay of blocking of PEDV binding to pAPN, anti-pAPN polyclonal antibody was prepared in rabbits using pAPN emulsified with Freund's adjuvant.

The method of Kessler [17] was used to prepare the brush border membrane. In brief, the small intestines of 10 days old piglets were collected and rinsed 7 times with cold saline. Mucosa was removed from the tissue by gentle scraping with the edge of slide glass. The tissue was placed in a volume (9 ml) equivalent to three times the weight of tissue (3 g) of mannitol buffer (2 mM Tris-HCl, 50 mM mannitol, leupeptin (1 µg/ml), pepstatin A (0.7 µg/ml), trypsin inhibitor (2.5 µg/ml), and 0.1 mM phenylmethanesulfonyl fluoride (PMSF)). The tissue was homogenized and diluted with five volumes of mannitol buffer (50 mM, pH 5.6) and homogenized once again. The final homogenate was incubated for 20 min on ice in the presence of 10 mM MgCl₂ and then centrifuged at 3,000 × g for 15 min. The supernatant was collected and centrifuged for 30 min at 27,000 × g. The pellet, representing the crude brush border membrane, was washed once by using the mannitol buffer and stored at -20°C until use. Porcine APN (pAPN) was purchased from Sigma (USA). The powder form of pAPN was rehydrated and diluted to optimal concentrations for each experiment with phosphate buffered saline (PBS, pH 7.4) for each experiment. All protein quantifications were performed by using BCA protein assay kit (Pierce, USA) according to the manufacturer's instruction.

To identify cellular proteins involved in PEDV binding, VOPBA was carried out. In brief, membrane proteins of cells were separated by SDS-PAGE. Cellular membranes of porcine brush border, ST, Vero, and PK-15 cells were boiled in 4X nonreducing sample buffer (4% sodium dodecyl sulfate, 10% glycerol, 0.625 M Tris-HCl, pH 6.8) and loaded on 8.5% polyacrylamide gels. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (PVDF, NEN Life Science, USA) at 45 V for 17 hours at 4°C in a buffer containing 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Nonspecific binding sites were blocked by incubating the membrane in PBS containing 5% skim milk, 1% bovine serum albumin, and 0.05% Tween 20 for 1 h. The membranes were incubated for 1 h with PEDV (10^{5.5} TCID₅₀/ml) or MEM, as a negative control, containing 20 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid) and 0.2% (w/v) sodium bicarbonate. The PVDF membrane was washed three times for 5 min each with PBS containing 0.05% Tween 20 (PBST), and incubated with normal mouse serum or PEDV monoclonal antibody. After washing three times

with PBST, horse-peroxidase labeled goat anti-mouse IgGs (KPL, USA), diluted to 1 : 5,000 in PBST, were added and incubated for 1 h at 37°C. Finally, substrate (ECL Western blotting detection reagents, Amersham, USA) was added. Developing was performed on the ECL film (Amersham, USA). As a control, VOPBA of TGEV was performed in porcine enterocytes using the same method as VOPBA of PEDV.

To detect the binding of PEDV to pAPN, direct virus-binding studies were carried out by enzyme linked immunosorbent assay (ELISA). A micro-ELISA plate (Nalge Nunc International, USA) was coated with 0.5 µg of pAPN per well in carbonate-bicarbonate buffer (pH 9.6). After overnight incubation at 4°C, it was washed 5 times with PBST. Blocking step was done using 3% gelatin in PBST. After washing, 10-fold serial diluted PEDV infected cell lysate (10^{5.5} TCID₅₀/0.1 ml) or mock infected cell lysate with PBST was added in 100 µl volumes, and incubated for 60 min at 37°C. Before the binding assay, PEDV and mock infected medium had been centrifuged 12,000 × g for 30 min to remove cell debris. The plates were washed and subsequently incubated with 100 µl of 1 : 50 diluted PEDV monoclonal antibody at 37°C for 60 min. The plates were washed and further incubated with 100 µl of horse-peroxidase labeled goat anti-mouse IgGs (KPL, USA) for 60 min. After washing the plate, ABTS substrate (2 mM 2,2-azino-di-3-ethyl-benzthiazole-sulfonate in 20 mM acetate (pH 4.2) plus 2.5 mM H₂O₂) solution was added and incubated for 20 min at room temperature. The reactions were stopped using 0.5 M H₂SO₄ and optical density was measured at 405 nm.

To test blocking activity of anti-pAPN for binding of PEDV to pAPN, pAPN coated plates were incubated with 10-fold serially diluted rabbit anti-pAPN polyclonal antibody or with normal rabbit serum for 60 min at 37°C. The remaining steps of the ELISA test were carried out as described above.

The effects of pAPN on PEDV replication were investigated in Vero cells. KPEDV-9 infected Vero cells were grown with A-VM in an experiment I. Vero cells were pretreated with pAPN before PEDV inoculation in an experiment II. As controls, KPEDV-9 was propagated in T-VM as described in a previous study [22].

In the experiment I, after inoculation with PEDV at a dose of 10^{3.7} TCID₅₀, Vero cells were incubated in the A-VM with pAPN concentrations ranging 0.024 µg/ml to 2.4 µg/ml. In the experiment II, Vero cell cultures were pretreated with pAPN at the concentrations ranging from 10 ng/ml to 1 mg/ml for 1, 2, or 3 h at 37°C. The cultures were washed three times with PBS and inoculated with PEDV at a dose of 10^{3.7} TCID₅₀. After adsorption at 37°C for 1 h, the cultures were washed three times with PBS and fed with VM. Virus showing 80% cytopathic effect (CPE) in both experiments was harvested and titrated.

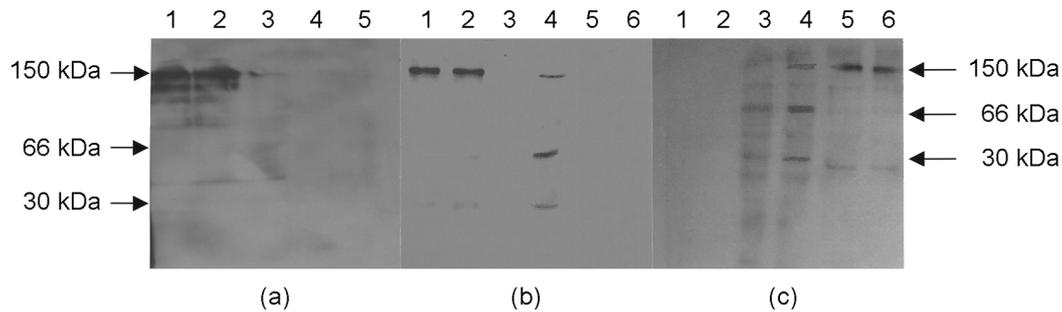


Fig. 1. Virus overlay protein binding assay. (a) TGEV using monoclonal antibody. Lane 1,2 porcine enterocytes, Lane 3-ST cells, Lane 4-Vero cells, Lane 5-negative control. (b) PEDV using monoclonal antibody. Lane 1,2-porcine enterocytes, Lane 3-Vero cells, Lane 4-ST cells, Lane 5, 6-negative control. (c) PEDV using polyclonal antibody. Lane 1-negative control, Lane 2-Vero cells, Lane 3,4-ST cells, Lane 5,6-porcine enterocyte.

To define the effects of pAPN in Vero cells, one-step growth curve of PEDV was carried out as described previously [15]. In an experiment III, the monolayered Vero cells in 6 well multiplates (Falcon, N.J., USA) were washed with PBS and inoculated with 1 ml of PEDV ($10^{3.5}$ TCID₅₀/ml) for 1 h at 37°C. After infection with PEDV into Vero cells, the cells were incubated with A-VM containing 2.4 µg/ml of pAPN.

In an experiment IV, the confluent monolayers of Vero cells were washed with PBS and treated with 10 µg/ml of pAPN for 1 h. After washing with PBS, Vero cells were inoculated with 1 ml of PEDV ($10^{3.5}$ TCID₅₀/), and adsorbed for 1 h at 37°C. After adsorption, monolayers were washed twice with PBS and incubated with 2 ml of VM without trypsin. As a control, T-VM was added to the plates which did not pretreat with pAPN.

All cell cultures were incubated at 37°C for 0, 2, 4, 6, 8, 10, 12, 15, 18, 24, 48 and 72 h. At the end of each incubation period, the media were harvested, centrifuged at 1,500 rpm for 10 min, and supernatants were stored at 4°C for the titration of extracellular (EC) PEDV. For intracellular (IC) virus, the cell pellets were resuspended in 2 ml of fresh VM. Cells still adhering to the bottom of the plate were washed twice with fresh VM, scraped off with a cell scraper (Costar, USA), then frozen and thawed three times to release IC virus particles. The ensuing suspension was clarified by centrifugation and titrated.

The virus titration was carried out at the 96 well microplate using Vero cells as described previously [21]. PEDV propagated with VM, A-VM or T-VM was diluted to serial ten-folds with VM. Confluent Vero cells were washed three times with PBS and inoculated with 0.1 ml inoculum into 5 wells each. Following adsorption for 1 h at 37°C, the inocula were removed and the monolayers were washed three times with PBS. Then, 0.1 ml of T-VM was added to each well and the cultures were incubated for 5 days at 37°C. Fifty % tissue culture infective doses (TCID₅₀) were expressed as the reciprocals of the highest virus dilution showing CPE.

The PEDV binding protein was detected in porcine enterocytes and ST cells. Interestingly, PEDV bound to a 150 kDa protein in porcine enterocyte. However, PEDV binding to a 66 kDa band was more dominant rather than that to a 150 kDa band in ST cells. No PEDV binding proteins were detected in Vero cells (Fig. 1).

As a positive control in the VOPBA, a receptor of TGEV was detected. The 150 kDa TGEV-specific binding protein was identified in porcine enterocytes and ST cells by using TGEV monoclonal antibody.

A series of virus-binding studies was performed to characterize the receptor-ligand interactions of KPEDV-9. The amount of pAPN-bound virus was determined directly by measuring the optical density. Data from absorbance showed that the binding of KPEDV-9 to pAPN was dose-dependent and increased to $10^{5.5}$ TCID₅₀/0.1 ml at 0.5 ng of pAPN concentration (Fig. 2). However, the saturation of PEDV binding was not reached under the condition employed. Mock infected media was not bound to pAPN. The binding of PEDV to pAPN could be blocked by rabbit anti-pAPN polyclonal antibody up to 1:10,000 dilutions in ELISA.

After treatment of soluble pAPN and trypsin, each virus was harvested when the 80% CPE was observed. PEDV was proliferated significantly by addition of soluble pAPN.

In the experiment I, infectious titers of PEDV grown in A-VM ranged from $10^{5.1}$ TCID₅₀/0.1 ml at 2.4~0.024 µg/ml of pAPN concentrations. The maximum PEDV titer was $10^{5.3}$ TCID₅₀/0.1 ml in the A-VM at 2.4 µg/ml of pAPN concentration. As controls, the titers of PEDV were $10^{4.1}$ TCID₅₀/0.1 ml in T-VM, and $10^{1.0}$ TCID₅₀/0.1 ml in VM without trypsin and pAPN (Fig. 3).

In the experiment II, the harvested PEDV grown in pAPN pretreated Vero cells ranged $10^{4.7-5.9}$ TCID₅₀/0.1 ml according to the concentration of pAPN. However, PEDV grown in T-VM was $10^{4.7}$ TCID₅₀/0.1 ml. The titer of PEDV cultured in VM without trypsin was $10^{2.5}$ TCID₅₀/0.1 ml in Vero cells which was not pretreated with pAPN.

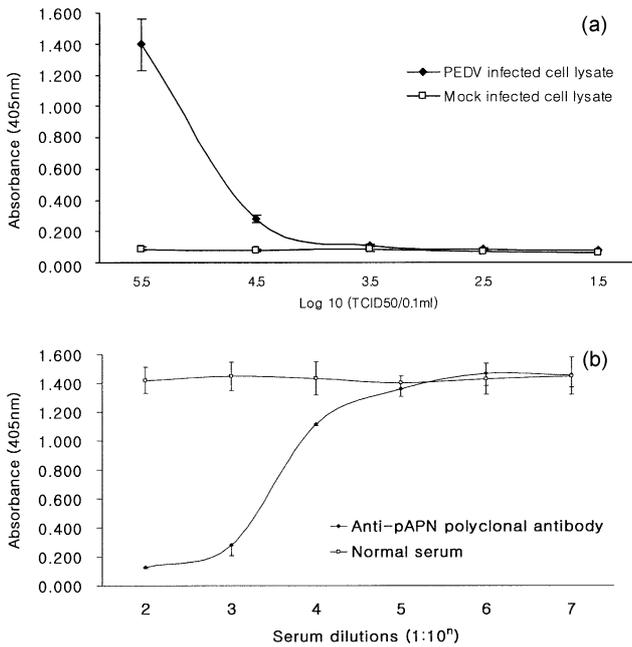


Fig. 2. PEDV binding activity to pAPN in ELISA. The micro-ELISA plate was coated at 0.5 ng of pAPN concentration per well. (a) Binding activities between PEDV and pAPN. The titer of PEDV-infected cell lysate was 10^{5.5} TCID₅₀/0.1 ml. (b) Blocking of PEDV binding to pAPN by an anti-pAPN antibody.

The maximum PEDV titer was obtained with 10^{5.9} TCID₅₀/0.1 ml at 10 μg/ml concentration of pAPN on Vero cell before PEDV inoculation. The best time of pAPN treatment was determined with 1 h at the concentration of pAPN employed (Fig. 4).

The virus growth patterns were very similar in both experiments. In the experiment III, PEDV was cultured with pAPN (2.4 pg/ml) simultaneously in VM. The replication kinetics is illustrated in Fig. 5. Intracellular (IC) PEDV growth patterns were very similar to those of A-

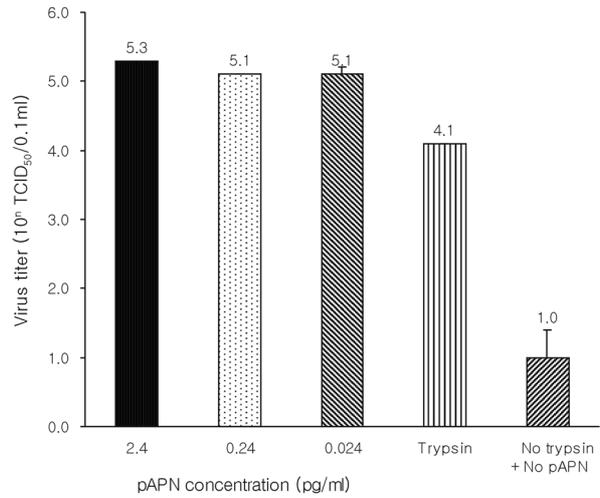


Fig. 3. PEDV infectivity in Vero cell cultured with pAPN simultaneously (Experiment I). The viral titers of PEDV were described in Mean ±S.D.

VM and T-VM. From six h after adsorption, the amount of IC PEDV began to increase and reached the peak between 24 and 48 h. The extracellular (EC) PEDV was released into media from 6 h after adsorption and peaked at 48 h in A-VM. In T-VM, however, the EC PEDV was released from 8 h and peaked at 48 h. The titer trends of EC PEDV were different during all incubation times. The virus yield of EC in A-VM (10^{5.0} TCID₅₀/0.1 ml) was higher as much as 10^{1.5} folds than that in T-VM (10^{3.5} TCID₅₀/0.1 ml) at 48 h after adsorption.

In the experiment IV, Vero cells were pretreated with pAPN (10 μg/ml) for 1 h before PEDV adsorption. The virus growth kinetics is illustrated in Fig. 5. From 4 h after adsorption, the amount of IC PEDV began to increase and reached the peak at 48 h. The EC PEDV was released into media from 6 h after adsorption and peaked at 48 h in

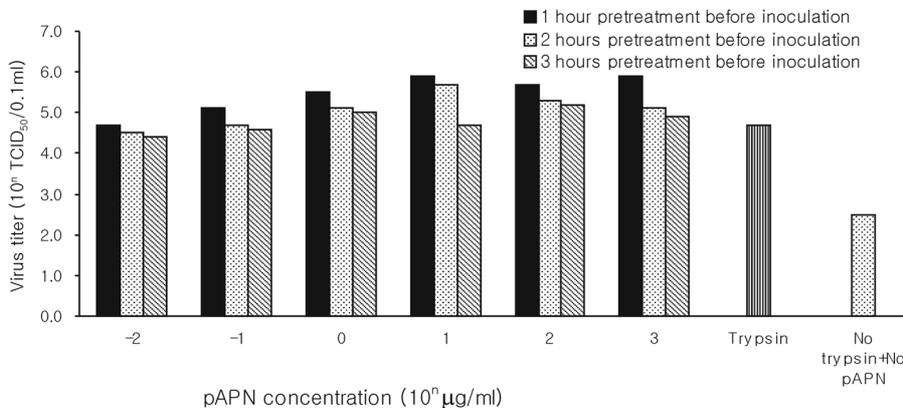


Fig. 4. PEDV infectivity in Vero cell pretreated with pAPN before inoculation (Experiment II). PEDV was cultured in virus replication medium without trypsin in pAPN pretreatment group.

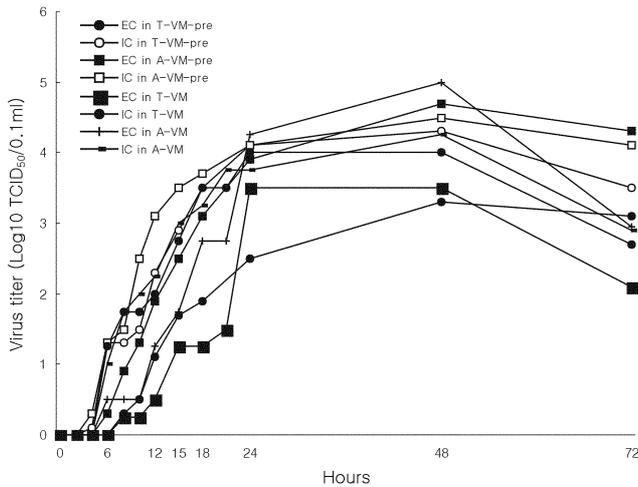


Fig. 5. One-step growth curve of PEDV cultured in Vero cells pretreated with pAPN before inoculation (Experiment IV) and inoculated with pAPN (Experiment III). EC: Extracellular PEDV, IC: Intracellular PEDV, A-VM: Virus replication medium with pAPN, T-VM: Virus replication medium with trypsin, pre: pretreated with pAPN before inoculation.

pAPN treatment. In T-VM, however, the EC PEDV was released from 8 h and peaked at 48 h. The titer trends of EC PEDV were different during the all incubation times. The virus yield of EC in pAPN treatment ($10^{4.7}$ TCID₅₀/0.1 ml) was higher as much as $10^{1.4}$ folds than that in T-VM ($10^{3.3}$ TCID₅₀/0.1 ml) at 48 h after adsorption. Moreover, the titer of EC PEDV cultured in VM was higher compared to that of EC PEDV cultured in T-VM.

The PEDV replicates in enterocytes of suckling pigs and causes ultrastructural changes mainly in the cytoplasm of enterocytes [16]. Using VOPBA, the authors identified a 150 kDa binding protein of PEDV in porcine enterocytes and the PEDV binding to pAPN could be demonstrated by ELISA using PEDV monoclonal antibody. In addition, this binding could be blocked by anti-pAPN antibody.

In a similar disease, pAPN is known as receptor for TGEV. APN is an 150 kDa ectoenzyme which is abundantly expressed at the apical membrane of the enterocytes. There were increasing evidences that APN is a common receptor for coronavirus group I [6,29]. Interestingly, feline APN (fAPN) acts as a common receptor for coronavirus in group I, whereas human and porcine APN glycoproteins serve only for human and porcine coronaviruses, respectively [29]. These facts lead to the speculation that PEDV may gain entry into the enterocytes through APN which is an 150 kDa ectoenzyme. But because of the lack of permissiveness of the APN-expressing porcine cell lines, it has been very difficult to confirm the receptor of PEDV. One of the most convincing methods of receptor identification is to transfect a putative receptor gene into a cell line

(nonpermissive cell line) to which the virus can not bind and demonstrate that the cell acquires the ability to bind virus and be infected through it. Another method, such as VOPBA, has also been used to identify receptor [2]. By using this method, the APN was identified as the receptor of TGEV [7]. By using VOPBA, a binding protein of PEDV was identified in porcine enterocytes and ST cells. In addition, APN was detected in ST cells and porcine enterocytes (not in Vero cells) by anti-APN monoclonal antibody (Data not shown). These results suggested that VOPBA was a useful screening procedure for identifying a virus receptor. A similar assay had been used successfully to identify putative receptors for several viruses including reovirus, Sendai virus, MHV-A59, Theiler's murine encephalomyelitis virus, echovirus, and cytomegalovirus [1,2,4,13,19,24,28,30]. The proteins of cells or their membranes were separated by SDS-PAGE, blotted, and overlaid with virus to determine whether virus could bind to any of the separated proteins [14].

As a positive control of VOPBA, the 150 kDa specific binding protein to TGEV was detected in porcine enterocytes and ST cells. Also the authors could detect the 150 kDa binding protein specific to PEDV in porcine enterocytes and about 66 kDa binding protein in ST cell. The distinction of specific proteins of PEDV in enterocyte and ST cells in size was supposed to allow the difference of permissiveness. But, inability of PEDV to replicate in ST cells suggests that there may be other factors required for virus replication likewise in Vero cells as well [31].

Although PEDV was replicated in Vero cell, the specific binding proteins to PEDV were impossible to be identified. Therefore, at present, the replication of PEDV in Vero cell could be explained as the following reasons. First, the trypsin, added to virus replication media when PEDV is cultured, may change the cell membrane so that the virus can bind to the cell membrane. As other coronaviruses like infectious bronchitis virus (IBV) and murine coronavirus, proteolytic cleavage of peplomeric glycoproteins may play an important role in the function of viral glycoprotein [20,27]. This cleavage is required for the activation of cell-fusing or neuraminidase activity [23]. Second, the attachment of virus to cell receptor may not be the only essential step for a virus to infect a target cell. In fact, neurotropic murine coronavirus has undergone cell receptor-independent infection [12]. This may suggest that PEDV infection in Vero cells is probably not mediated by an interaction between the virus and a relevant receptor. Because Vero cells are widely used to grow heterologous viruses, it could be assumed that broad permission of virus in Vero cells is probably due to an intrinsic property of the cells, and not due to the presence of a receptor.

In this study, the authors showed that binding of PEDV to pAPN was dose-dependent and blocked by anti-pAPN antibody. However, saturation of PEDV binding was not

reached under the condition used because the virus titers exceeding $10^{5.5}$ TCID₅₀/0.1 ml could not prepare in PEDV propagation. As a similar study, porcine reproductive and respiratory syndrome virus (PRRSV) bound specifically to alveolar macrophage in a dose-dependent manner [25].

One step growth curve for PEDV showed that the virus, which was cultured in Vero cell with simultaneous treatment or pretreatment with APN, yields higher titers than that in T-VM. Especially, in pAPN-pretreated Vero cell, EC PEDV showed higher titers compared to that in T-VM. However, there was no synergism with trypsin and pAPN. Because soluble form of the HIV receptor CD4 could enhance the infection of cells by CD4-induced fusogenic conformational changes of the envelope glycoproteins [26], our data indicated that PEDV might bind by means of pAPN and induced fusogenic conformation for promoting infection in Vero cells of PEDV. Another explanation of increased PEDV titer in Vero cells treated with pAPN is that pAPN could play a role as a cofactor for the replication of PEDV. In human immunodeficiency virus infection, binding of the gp120 envelope glycoprotein to the CD4 receptor was not sufficient in itself to allow virus entry, and additional components in the membrane were required for cell infection as a cofactor, serine protease named tryptase TL2, in the membrane of CD4+ lymphocytes [18].

Conclusively, the authors demonstrated that PEDV bound 150 kDa protein in enterocytes using VOPBA. The PEDV binding to pAPN was blocked by anti-pAPN antibody. It supports that soluble form of pAPN could increase the virus yield in cell culture. These results might suggest that pAPN plays an important role in infection and replication of PEDV in enterocytes.

Acknowledgment

This work was supported by the 2000 University-Industry Cooperative Activities Program of Korea Science and Engineering Foundations (Grant#2000-22200-001-1), the Brain Korea 21 Project, and the Research Institute for Veterinary Science, Seoul National University.

References

1. **Borrow, P. and Olstone, M. B. A.** Characterization of lymphocytic choriomeningitis virus-binding protein(s). A candidate cellular receptor for the virus. *J. Virol.* 1992, **66**, 7270 - 7281.
2. **Boyle, J. F., Weismiller, D. G. and Holmes, K. V.** Genetic resistance to mouse hepatitis virus correlates with absence of virus-binding activity on target tissues. *J. Virol.* 1987, **61**, 185 -189.
3. **Bridgen, A., Kocherhans, R., Tobler, K., Carvajal, A. and Ackermann, M.** Further analysis of the genome of porcine epidemic diarrhea virus. *Adv. Exp. Med. Biol.* 1998, **440**, 781-786.
4. **Dalziel, R. G., Hopkins, J., Watt, N. J., Dutia, B. M., Clarke, H. A. K. and McConnell, I.** Identification of a putative cellular receptor for the lentivirus visna virus. *J. Gen. Virol.* 1991, **72**, 1905-1911.
5. **de Bouck, P., Pensaert, M. and Coussement, W.** The pathogenesis of an enteric infection in pigs, experimentally induced by the coronavirus-like agent, CV777. *Vet. Microbiol.* 1981, **6**, 157-165.
6. **Delmas, B., Gelfi, J., L'Haridon, R. and Sjostrom, H.** Further characterization of aminopeptidase N as a receptor for coronavirus. *Adv. Exp. Med. Biol.* 1994, **342**, 293-298.
7. **Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L.K., Sjostrom, H., Noren, O. and Laude, H.** Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. *Nature* 1992, **357**, 417-420.
8. **Duarte, M. and Laude, H.** Sequence of the spike protein of the porcine epidemic diarrhea virus. *J. Gen. Virol.* 1994, **75**, 1195-1200.
9. **Duarte, M., Tobler, K., Bridgen, A., Rasschaert, D., Ackermann, M. and Laude, H.** Sequence analysis of the porcine epidemic diarrhea virus genome between the nucleocapsid and spike protein genes reveals a polymorphic ORF. *Virology* 1994, **198**, 466-476.
10. **Ducatelle, R., Coussement, W., Pensaert, M., de Bouck, P. and Hoorens, J.** *In vivo* morphogenesis of a new porcine enteric coronavirus, CV777. *Arch. Virol.* 1981, **68**, 35-44.
11. **Enserink, M.** Calling all coronavirologists. *Science* 2003, **300**, 413-414.
12. **Gallagher, T. M., Buchmeier, M. J. and Perlman, S.** Cell receptor-independent infection by a neurotropic murine coronavirus. *Virology* 1992, **191**, 517-522.
13. **Gershoni, J. M., Lapidot, M., Zakai, N. and Loyter, A.** Protein blot analysis of viral receptors: Identification and characterization of the Sendai virus receptor. *Biochim. Biophys. Acta.* 1986, **856**, 19-26.
14. **Haywood, A. M.** Virus receptors: binding, adhesion strengthening, and changes in viral structure. *J. Virol.* 1994, **68**, 1-5.
15. **Hoffman, M. and Wyler, R.** Propagation of the virus of porcine epidemic diarrhea in cell culture. *J. Clin. Microbiol.* 1988, **26**, 2235-2239.
16. **Horvath, I. and Moscardi, E.** Ultrastructural changes in the small intestinal epithelium of suckling pigs affected with transmissible gastroenteritis (TGE)-like disease. *Arch. Virol.* 1981, **68**, 103-113.
17. **Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M. and Semenza, G.** A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membrane. *Biochim. Biophys. Acta.* 1978, **506**, 136-154.
18. **Kido, H., Niwa, Y., Beppu, Y. and Towatari, T.** Cellular proteases involved in the pathogenicity of enveloped animal viruses, human immunodeficiency virus, influenza virus A and Sendai virus. *Adv. Enzyme Regul.* 1996, **36**, 325-347.
19. **Kilpatrick, D. R. and Lipton, H. L.** Predominant binding of Theiler's viruses to a 34-kilodalton receptor protein on susceptible cell lines. *J. Virol.* 1991, **65**, 5244-5249.

20. **Klenk, H. D. and Rott, R.** Cotranslational and posttranslational processing of viral glycoproteins. *Curr. Top. Microbiol. Immunol.* 1980, **90**, 19-48.
21. **Kusanagi, K., Kuwahara, H., Katoh, T., Nunoya, T., Ishikawa, Y., Samejima, T. and Tajima, M.** Isolation and serial propagation of porcine epidemic diarrhea virus infection in cell cultures and partial characterization of the isolate. *J. Vet. Med. Sci.* 1992, **54**, 303-318.
22. **Kweon, C. H., Kwon, B. J., Lee, J. G., Kwon, G. O. and Kang, Y. B.** Derivation of attenuated porcine epidemic diarrhea virus (PEDV) as vaccine candidate. *Vaccine* 1999, **17**, 2546-2553.
23. **Lazarowitz, S. G. and Choppin, P. W.** Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of hemagglutinin polypeptide. *Virology* 1975, **68**, 440-454.
24. **Mbida, A. D., Pozzetto, B., Gaudin, O. G., Grattard, F., Bihan, J-C. L., Akono, Y. and Ros, A.** A 44,000 glycoprotein is involved in the attachment of echovirus-11 onto susceptible cells. *Virology* 1992, **189**, 350-353.
25. **Nauwynck, H. J., Duan, X., Favoreel, H. W., van Oostveldt, P. and Penasert, M. B.** Entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages via receptor-mediated endocytosis. *J. Gen. Virol.* 1999, **80**, 297-305.
26. **Schenten, D., Marcon, L., Karlsson, G. B., Parolin, C., Kodama, T., Gerard, N. and Sodroski, J.** 1999. Effect of soluble CD4 on simian immunodeficiency virus infection of CD4-positive and CD4-negative cells. *J. Virol.* 1999, **73**, 5373-5380.
27. **Sturman, L. S., Ricard, C. S. and Holmes, K. V.** Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: activation of cell-fusing activity of virions by trypsin and separation of two different 90K cleavage fragments. *J. Virol.* 1985, **56**, 904-911.
28. **Taylor, H. P. and Cooper, N. R.** The human cytomegalovirus receptor on fibroblasts is a 30-kilodalton membrane protein. *J. Virol.* 1990, **64**, 2484-2490.
29. **Tresnan, D. B. and Holmes, K. V.** Feline aminopeptidase N is a receptor for all group I coronaviruses. *Adv. Exp. Med. Biol.* 1998, **440**, 69-75.
30. **Verdin, E. M., King, G. L. and Maratos-flier, E.** Characterization of a common high affinity receptor for reovirus serotypes 1 and 3 on the endothelial cells. *J. Virol.* 1989, **63**, 1318-1325.
31. **Xue, W. and Minocha, H.C.** 1996. Identification of bovine viral diarrhea virus receptor in different cell types. *Vet. Microbiol.* 1996, **49**, 67-79.