Porcine aminopeptidase N is not a cellular receptor of porcine epidemic diarrhea virus, but promotes its infectivity via aminopeptidase activity

Kazuya Shirato,1 Madoka Maejima,1 Md. Taimur Islam,2 Ayako Miyazaki,3 Miyuki Kawase,1 Shutoku Matsuyama1 and Fumihiro Taguchi1,2

1Laboratory of Acute Respiratory Viral Diseases and Cytokines, Department of Virology III, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan
2Laboratory of Virology and Viral Infections, Faculty of Veterinary Medicine, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino, Tokyo 180-8602, Japan
3Viral Diseases and Epidemiology Research Division, National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

Porcine epidemic diarrhea virus (PEDV), a causative agent of pig diarrhoea, has recently caused significant economic damage worldwide. Porcine aminopeptidase N (pAPN) has been reported to be the receptor for PEDV, although robust evidence is lacking. In the present study, we explored whether pAPN functions as a receptor for PEDV. Human HeLa cells expressing pAPN and pAPN-positive porcine CPK cells failed to support PEDV infection, but were susceptible to infection by transmissible gastroenteritis virus (TGEV), which utilizes pAPN as a functional receptor. In contrast to TGEV, PEDV did not bind soluble porcine aminopeptidases (pAPs) and infection was not inhibited by the soluble form of pAPs. However, overexpression of pAPN in porcine CPK cells (CPK-pAPN cells) slightly increased the production of PEDV, and the increased replication in CPK-pAPN cells was inhibited by bestatin, an inhibitor of the protease activity of aminopeptidase N. These results suggest that pAPN is not a functional receptor for PEDV, but promotes the infection of PEDV through its protease activity.

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is a member of the genus Alphacoronavirus, which is an enveloped virus containing single-stranded, positive-sense genomic RNAs of about 30 kb in size (Lai & Cavanagh, 1997). PEDV, a causative agent of pig diarrhoea, induces loss of appetite and weight loss in adult pigs and is lethal in piglets (Pensaert & de Bouck, 1978). PEDV was first described in Europe in the 1970s (Chasey & Cartwright, 1978; Pensaert & de Bouck, 1978). Thereafter, PEDV spread to Asian countries and caused recurrent problems therein, severely reducing pig farming success because of the high piglet mortality (Chen et al., 2008; Kusanagi et al., 1992; Li et al., 2012; Park et al., 2007; Puranaveja et al., 2009; Sun et al., 2012; Vui et al., 2014). North America was PEDV-free until May 2013, except for one suspected case in Canada (Turgeon et al., 1980). However, an outbreak occurred in Ohio in the USA, subsequently spreading throughout the entire USA and resulting in significant economic damage (Chen et al., 2014; Huang et al., 2013; Wang et al., 2014).

Coronaviruses (CoVs) are species-specific pathogens that cause various diseases, generally growing in cultured cell lines established from susceptible animal species (Weiss & Navas-Martin, 2005). A major determinant of species-specificity is the cellular receptor; that is, the molecule with which the virus initially interacts on the cell surface (Holmes & Compton, 1995). Some alpha CoVs, including human coronavirus 229E (HCoV-229E), canine coronavirus and feline enteric coronavirus serotype 2, and another enteropathogenic porcine coronavirus [transmissible gastroenteritis virus (TGEV)] utilize the aminopeptidase N (APN) of the host animal species as a functional receptor, i.e. expression of APN allowed cells to bind virions and rendered cells to be permissive for the infection (Delmas et al., 1992; Tresnan et al., 1996; Yeager et al., 1992). APN is found in a variety of tissues; in particular, a high concentration is found in epithelial cells of the small intestine and kidney (Delmas et al., 1994; Kenny & Maroux, 1982; Semenza, 1986).
To date, compelling evidence on the nature of the cellular receptor of PEDV is lacking. Some authors reported that porcine APN (pAPN), the TGEV receptor, was a cellular receptor for PEDV (Li et al., 2007; Nam & Lee, 2010; Oh et al., 2003). For example, Oh and colleagues reported that pAPN expression in Vero cells enhanced viral replication, as did the addition of soluble APN to culture medium. However, the authors did not explore whether PEDV used expressed pAPN (Oh et al., 2003). In addition, Li and colleagues used an immunofluorescence assay to demonstrate that PEDV antigen could be detected in PEDV-inoculated pAPN-expressing Mardin–Darby canine kidney (MDCK) cells. However, viral multiplication in such cells was not clearly demonstrated (Li et al., 2007). Park and colleagues reported that transgenic mice expressing pAPN showed virus susceptibility following experimental infection, although immunohistochemical evaluation of control mice resulted in an antigen-positive signal (Park et al., 2015). Most importantly, all of those studies lacked a positive control; they did not examine the susceptibility of cells to TGEV, which has been clearly shown to utilize pAPN as a functional receptor (Delmas et al., 1992; Tresnan et al., 1996; Yeager et al., 1992). Thus, there is no robust evidence that PEDV infects cells expressing pAPN. In addition, the fact that PEDV infects and replicates in pAPN-negative Vero cells (Hofmann & Wyler, 1988; Kusanagi et al., 1992) suggests that pAPN is not a functional receptor for PEDV, because Vero cells are established from green monkey kidney cells and do not express pAPN. Recently, Meng et al. (2014) reported that a peptide that recognized pAPN inhibited PEDV infection in Vero cells, which was independent of the interaction with pAPN, and interacted with PEDV S protein. This study suggests that a molecule closely related to pAPN (but different from pAPN) acts as a receptor for PEDV infection.

In the present study, we explored whether pAPN acts as a functional receptor for PEDV using a positive control, TGEV, in most analyses. Our results suggest that pAPN does not act as a receptor of PEDV, but does act as a functional receptor of TGEV. In addition, we show that pAPN promotes PEDV infection in some cell lines via its protease activity.

RESULTS

PEDV replication in pAPN-expressing HeLa cells

To explore whether the expression of pAPN in non-susceptible cells allows for PEDV replication, a clone of human-derived HeLa cells stably expressing pAPN was established after transfection with the pAPN gene. The expression levels of pAPN mRNA in the cloned cells were determined using a real-time PCR assay (Fig. 1a). pAPN mRNA was detected only in HeLa-pAPN and CPK cells, but not in HeLa or Vero cells. The expression level of pAPN mRNA in HeLa-pAPN cells was about 170-fold higher than that in CPK cells. PEDV replication was then determined using HeLa-pAPN cells with TGEV as a positive control (Fig. 1b). TGEV replicated to high levels in both CPK and HeLa-pAPN cells expressing pAPN, attaining a peak titre of $10^6$ TCID$_{50}$ (100 µl)$^{-1}$, but did not replicate in pAPN-negative cells, confirming previous reports that TGEV utilized pAPN as a cellular receptor (Delmas et al., 1992; Tresnan et al., 1996; Yeager et al., 1992). In contrast, PEDV infected and replicated in pAPN-negative Vero cells regardless of trypsin treatment, though the efficiency was low in the absence of trypsin, and trypsin treatment enhanced PEDV replication approximately 100-fold. These results were in good agreement with our previous report (Shirato et al., 2011). However, PEDV failed to replicate in pAPN-expressing cells, namely, HeLa-pAPN and CPK cells in the absence of trypsin. In the presence of trypsin, PEDV showed very weak and transient replication in CPK cells, but not in HeLa-pAPN cells. Viral replication was also confirmed by antigen expression within cells (Fig. 1c). Two days after viral inoculation, cells were fixed and stained using specific antisera. Viral antigens were detected in CPK and HeLa-pAPN cells after TGEV infection. However, PEDV antigen was not observed in pAPN-positive HeLa-pAPN or CPK cells, but only in Vero cells, regardless of the presence or absence of trypsin in the culture medium. HeLa cells overexpressing a variety of CoV receptors, such as murine CEACAM1 and human ACE2, are susceptible to mouse hepatitis virus (MHV), severe acute respiratory syndrome (SARS)-CoV and HCoV-NL63 (Kawase et al., 2009, 2012; Watanabe et al., 2008). This cell line also became susceptible to TGEV when its receptor was expressed, as shown above. However, HeLa cells expressing pAPN were not susceptible to PEDV. It is possible that HeLa cells lack a critical cellular factor for the replication of PEDV. To explore this hypothesis, we examined whether HeLa cells permit PEDV replication by bypassing receptor-mediated entry of the viral genome, i.e. direct transfection of genomic RNA into the cells. We prepared highly concentrated and purified viral RNA, as described in Methods, and transfected this into HeLa cells. After 3 days of incubation, the cell lysates prepared from transfected cells were inoculated onto Vero cells and the infectivity of progeny virus was examined using an immunofluorescence assay (Fig. 2a). The lysate obtained from PEDV RNA-transfected Vero cells showed positive signals after inoculation onto Vero cells. This was also observed in Vero cells inoculated with viral RNA-transfected HeLa cell lysate, although the efficiency was lower in HeLa cells than that in Vero cells. The mean foci numbers in RNA-transfected cells were 19.4±6.8 (Vero) and 9.5±5.2 (HeLa) (Fig. 2b). Vero cells inoculated with the lysates of mock-transfected and PEDV-inoculated HeLa cells showed no virus infection. These results strongly suggest that HeLa cells are equipped to support PEDV infection if its genome can successfully enter into the cells, implying that PEDV infects HeLa cells expressing pAPN if pAPN functions as a receptor. These findings suggest that pAPN does not serve as a functional receptor for PEDV.
Recently, it was reported that not only pAPN but also human and monkey APN function as receptors of PEDV (Liu et al., 2015). The HeLa cells used in our study, which express human APN, are susceptible to 229E (Kawase et al., 2009). However, the HeLa cells failed to support PEDV infection, suggesting that human APN does not function as a receptor of PEDV.

**Role of soluble pAPN in PEDV replication**

In a variety of virus species, such as CoVs, retroviruses and poliovirus, pretreatment with soluble receptor neutralizes viral infectivity (Balliet et al., 1999; Breslin et al., 2003; Hussey et al., 1988; Kaplan et al., 1990; Saeki et al., 1997). If pAPN is an authentic receptor for PEDV, its soluble form...
should neutralize the virus, as described above. We examined whether pretreatment with soluble pAPN [porcine aminopeptidase (pAP)] neutralizes the infectivity of PEDV toward Vero cells. We used TGEV as a control as it utilizes pAPs as a receptor (Delmas et al., 1992; Tresnan et al., 1996; Yeager et al., 1992). PEDV and TGEV were pretreated with the indicated concentrations of pAPs for 60 min and allowed to infect Vero or CPK cells. Virus infection was monitored by real-time PCR using primers for the subgenomic mRNA of the N protein (Fig. 3). PEDV infection was not blocked by pAPs, suggesting that soluble pAPN failed to neutralize PEDV. In contrast, TGEV infection was blocked by the pAPs in a dose-dependent manner, confirming that soluble pAPN neutralized TGEV infection. These findings, in addition to the above observations, indicate that pAPN functions as a receptor for TGEV, but not for PEDV.

**Evaluation of PEDV binding to pAPN**

The data obtained in the above analyses suggested that pAPN did not function as a receptor for PEDV. Therefore, we explored whether pAPN binds to PEDV. To accomplish this, we developed a co-precipitation assay using hydrophobic magnetic beads. As described in Methods, Dynabeads M-270 epoxy magnetic beads were mixed with pAPs, and binding was confirmed via Western blotting (WB) (Fig. 4a). WB analysis showed the pAP contained two different molecules other than pAPN. The size of the cell-membrane-
bound pAP was about 150 kDa, and its soluble form was about 100 kDa (Breslin et al., 2003). The pAP used, L5006, is derived from microsomes of the porcine kidney, and is thought to contain various pAPs. Virions were then allowed to bind to the pAPs on the beads and the amounts were determined using real-time RT-PCR with a probe against genomic RNAs (Fig. 4b). TGEV bound to pAP-beads at levels 10-fold greater than control beads, whereas PEDV failed to bind pAPs on the beads. This result is in good agreement with our previous finding that pAPN is not a receptor of PEDV.

Receptor competition experiments

If PEDV and TGEV use the same molecule as a cellular receptor, the infectivity of these viruses during a mixed infection will be influenced by receptor competition. Therefore, a competitive infection assay was performed (Fig. 5). A total of \(1 \times 10^5\) PEDV or TGEV were mixed with \(1 \times 10^5\) live or UV-inactivated TGEV or PEDV as a competitor virus and were then inoculated onto Vero or CPK cells, respectively. UV-inactivated TGEV and PEDV decreased each virus infection to the level of about 50%. However, PEDV and TGEV infections were not affected by the presence of competitor TGEV or PEDV, respectively, regardless of UV inactivation. This demonstrated that the infectivity of TGEV and PEDV was unaffected by the presence of the other virus in a mixed infection, suggesting that PEDV and TGEV use different molecules as cellular receptors.

Effect of overexpression of pAPN in CPK cells

Nam & Lee (2010) reported that pAP density was an important factor in PEDV infection based on experiments using swine testis (ST) cells. Therefore, CPK cells expressing approximately 1000-fold higher pAP levels than parental CPK cells were obtained by transfection with the pAPN.
gene (Fig. 6a), and the effect of pAPN expression on PEDV infection in porcine-derived cells was evaluated (Fig. 6c, d). Cells were inoculated with TGEV or PEDV and virus replication was determined after 3 days of incubation. The replication of TGEV was enhanced more than 10-fold by the overexpression of pAPN in CPK cells (Fig. 6b). PEDV replication was enhanced in CPK-pAPN cells in the presence of trypsin, although the titre was 100-fold lower than that in Vero cells (Fig. 6c). The enhanced replication was not seen in the absence of trypsin (Fig. 6d). These results suggest that pAPN promotes its replication through an unknown mechanism in CPK cells, in cooperation with trypsin.

Involvement of pAPN enzymatic activity in infection by PEDV, but not TGEV

As described above, PEDV replication was slightly enhanced in the presence of high concentrations of pAPN and trypsin in CPK cells. We then explored whether the enzymatic activity of pAPN was associated with the replication of PEDV in pAPN-overexpressing CPK cells using an aminopeptidase (AP) inhibitor, bestatin (ubenimex), that did not affect trypsin activity (Umezawa et al., 1976). As shown in Fig. 7(a), CPK-pAPN cells showed higher APN activity than that of the CPK cells, and the enzymatic activity of cell-membrane-bound APN was inhibited by 100 µM bestatin to a level similar to that of control CPK cells. Bestatin treatment did not affect cell viabilities within 3 days of incubation (Fig. 7b). Despite its use of pAPN as a cellular receptor, infection by TGEV was not reduced by treatment of cells with bestatin (Fig. 7c). In contrast, as shown in Fig. 7(d), PEDV showed higher replication in CPK-pAPN cells in the presence of trypsin than in CPK cells, identical to the data in Fig. 6(c). However, this increased replication was affected by bestatin; PEDV replication was decreased approximately 10-fold in CPK-pAPN cells by bestatin treatment (Fig. 7d), suggesting that the enzymatic activity of pAPN is responsible for its promotion of PEDV replication in CPK cells.

To evaluate whether the enhanced replication of PEDV was indeed dependent on the enzymatic activity of pAPN-overexpressed in CPK cells or not, an expression plasmid of an enzymatically inactive mutant of pAPN (pAPNmt) was prepared following Nam’s report (Nam & Lee, 2010) and stably expressed cells were cloned. The expression level of mRNA for pAPNmt was about 2740 times higher than that in CPK cells (Fig. 6a). The enzymatic activity in terms of pAPN was measured using alanine substrate and, as expected, the activity was lower than that in CPK-pAPN, and was similar to that in the parental CPK cells (Fig. 7a). The overexpression of pAPNmt increased TGEV replication similar to intact pAPN expression (Fig. 6b). The expression of pAPNmt certainly allowed PEDV to infect CPK cells in the presence of trypsin, although the titre was significantly lower than for infection in cells expressing intact pAPN (Fig. 6c, d). Bestatin treatment did not affect this very imperceptible increase of virus replication (Fig. 7d). These results show that pAPN enzymatic activity contributed to the infection of PEDV together with a not-yet-identified receptor.

DISCUSSION

pAPN was reported to act as the cellular receptor for PEDV (Li et al., 2007; Oh et al., 2003). This is not unexpected considering APN is highly expressed on epithelial cells of the small intestine (Kenny & Maroux, 1982) and PEDV infection is mostly restricted to intestinal epithelial cells (Kim & Chae, 2000). However, robust evidence that pAPN is the PEDV receptor is lacking. In most studies, Vero cells deprived of pAPN were used to support PEDV replication (Hofmann & Wyler, 1988; Kusanagi et al., 1992). Thus, the question of whether pAPN acts as a receptor for PEDV...
remained controversial. The present study was performed to determine whether pAPN is a robust receptor for PEDV. We found that expression of pAPN in HeLa cells failed to render the cells susceptible to PEDV, but they did become susceptible to TGEV, which is known to utilize pAPN as a functional receptor (Delmas et al., 1992).

Expression of the receptor molecules of other CoVs in HeLa cells rendered cells susceptible to several CoVs, such as SARS-CoV (Kawase et al., 2009; Watanabe et al., 2008), human coronavirus NL-63 (Kawase et al., 2012), MHV (Kawase et al., 2009) and feline infectious peritoneal virus (Würdinger et al., 2005). The present study also showed that pAPN expression in HeLa cells facilitated TGEV replication. These findings suggest that HeLa cells retain the machinery required for the replication of CoVs (even for PEDV), and that if an authentic PEDV receptor is expressed, they acquire susceptibility to these viruses. The idea that HeLa cells are equipped for full replication of PEDV was supported by the findings shown in the present study; infectious viruses were produced in HeLa cells following transfection of the PEDV genome. These results collectively support the notion that pAPN is not a functional receptor for PEDV.

Furthermore, we also found that PEDV could not bind to pAPs and was not neutralized by treatment with soluble pAPN, while TGEV could bind to pAPs and was neutralized. Furthermore, we found that PEDV infection was inhibited by the mixed infection of Vero cells with UV-inactivated PEDV, but not with live or UV-inactivated TGEV, indicating that the receptor for PEDV differs from

![Fig. 6.](image_url)

**Fig. 6.** The effect of overexpression of pAPN in CPK cells. (a) Expression plasmids containing pAPN and a drug selection marker were transfected into CPK cells, and pAPN-overexpressing clones were selected using G418 for CPK-pAPN and CPK-pAPNmt cells. pAPN mRNA levels were determined by real-time PCR assays using specific TaqMan probes. Data were normalized to those of 18S rRNA obtained from the same sample and are expressed as relative values compared to normal CPK cells (n=3). (b, c, d) Virus replication in CPK-pAPN and CPK-pAPNmt cells was estimated. TGEV and PEDV were inoculated onto the cells at an m.o.i. of 0.1. After virus adsorption, cells were incubated in 10% TPB-DMEM containing 1.25 µg trypsin ml⁻¹ for PEDV (c), and in the same medium without trypsin for TGEV (b) and PEDV (d). After 3 days, cells and supernatants were collected together, and titres were determined (n=6). Statistical significance is indicated in the graph.
pAPN is not a receptor for PEDV but works as a protease

Fig. 7. Effect of inhibition of pAPN protease activity by bestatin. (a) CPK, CPK-pAPN and CPK-pAPNmt cells were cultured in 96-well plates. APN activity was measured as described in Methods using alanine as a substrate. Serially diluted pAPs were used to generate a standard curve for calculation of APN activity. (b, c, d) CPK, CPK-pAPN and CPK-pAPNmt cells were incubated in 10% DMEM containing 100 μM bestatin for 60 min. After treatment, cells were infected with viruses in the presence of bestatin for 60 min. After virus adsorption, cells were incubated in 10% TPB-DMEM containing bestatin and 1.25 μg trypsin ml⁻¹ (for PEDV, d) or in 10% TPB-DMEM containing bestatin (for TGEV, c). After 3 days, cells and supernatants were collected together, and virus titres were determined (n=6). Cell viabilities in the presence of bestatin were determined by staining with 0.2% (w/v) trypan blue/PBS (b, n=4).
that for TGEV. These findings strengthened the notion that pAPN is not a functional receptor of PEDV.

Nam & Lee (2010) showed that the density or expression level of pAPN was important in terms of PEDV entry into cells because ST cells, which express relatively low levels of pAPN, were not susceptible to PEDV but were susceptible to TGEV, and overexpression of pAPN in ST cells allowed for PEDV infection. Cong et al. (2015) also reported that transient overexpression of pAPN in and pAPN small interfering RNA (siRNA) treatment of porcine small intestine epithelial cells increased and decreased the infectivity of PEDV, respectively. Therefore, in the present study pAPN was overexpressed in porcine CPK cells, and the effect on PEDV replication was examined. We found that overexpression of pAPN in CPK cells facilitated PEDV replication in CPK cells, which is in good agreement with the findings of Nam and Lee (2010) and the observations of Cong et al. (2015). Furthermore, we found that the enhanced replication was caused by the enzymatic activity of pAPN, because PEDV replication in CPK cells overexpressing pAPN was blocked by treatment with bestatin, a pan-AP inhibitor. In addition, the expression of an enzymatically inactive mutant of pAPN did not show a significant increase of PEDV replication as was shown for intact pAPN expression. These results indicate that although pAPN is not the receptor for PEDV, it may promote PEDV replication via its protease activity. It remains unclear how pAPN enzymatic activity facilitates PEDV infection in CPK cells, which should be the subject of further research.

To date, four different types of proteins have been identified as CoV receptors. Initially, carcinomaembryonic cell adhesion molecule 1 (CEACAM1) was found to act as a receptor for MHV (Williams et al., 1991), and thereafter, pAPN and human APN were reported to be receptors for TGEV (Delmas et al., 1992) and HCoV-229E (Yeager et al., 1992), respectively. Feline APN was also reported to be the receptor for feline, canine, porcine and human CoVs (Tresnzan et al., 1996). Receptors for the highly pathogenic human respiratory viruses SARS-CoV and Middle East respiratory syndrome (MERS)-CoV have been identified as angiotensin-converting enzyme 2 (ACE2) (Li et al., 2003) and dipeptidyl peptidase 4 (DPP-4) (Raj et al., 2013). We examined using real-time PCR whether pAPN cells susceptible to TGEV but not PEDV express receptors utilized by other CoVs, and found that pAPN cells express porcine homologues of CEACAM1, ACE2 and DPP-4 (data not shown), as well as pAPN, as shown in the present study. Thus, PEDV does not use the molecules identified so far as receptors of other CoVs, since pAPN cells are not susceptible to PEDV. It is possible that PEDV utilizes a molecule different from the receptors reported for other CoVs.

Sequence analysis of PEDV strains recently isolated in China (in 2012 and 2013) and recent US isolates showed that the new viruses are distinct from classical PEDV strains. A bootscan analysis suggested that the recent US strains were similar to Chinese strains isolated after 2011 (Huang et al., 2013). Most PEDVs, including classic and recent strains, have been isolated and propagated using Vero cells, as shown in many papers (Chen et al., 2014; Hofmann & Wyler, 1988; Kusanagi et al., 1992; Pan et al., 2012; Shibata et al., 2000; Vui et al., 2014). It is generally accepted that Vero cells are most suitable for PEDV infection. In addition, we also isolated PEDVs from piglets with diarrhoea in Japan in 2014, and the S proteins of those viruses showed 99% similarity to the S protein of the recent US isolates (Islam et al., 2016). These viruses multiplied in Vero cells, but failed to infect cells expressing pAPN, HeLa-pAPN (data not shown). This finding does not support the idea that the MK strain of PEDV used in the present study had affinity to pAPN initially but lost that affinity after passage in Vero cells, since even new isolates from infected animals failed to utilize pAPN as a functional receptor. It is supposed that not only classical PEDV but also recent isolates use a cell receptor other than pAPN. Identification of the authentic receptor is important for the development of a strategy applicable worldwide to prevent or cure porcine epidemic diarrhoea.

METHODS

**Cells.** Vero cells (CCL-81) were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA, USA). The HeLa cells used were HeLa-229 (ATCC-CCL2.1). CPK cells derived from porcine kidney were also used. The 293T/17 (ATCC-CCL 11268) cells were also obtained from the ATCC. The expression plasmid pCAGGS containing the pAPN sequence (pCAGGS-pAPN) was kindly provided by Dr Hide-toshi Ikeda, Nippon Veterinary and Life Science University (Tokyo, Japan). HeLa cells constitutively expressing pAPN and CPK cells overexpressing pAPN were constructed via co-transfection with pCAGGS-pAPN and pTargeT harbouring a neomycin-resistance gene (Promega); this enabled clonal selection with G418. All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) containing 5% (v/v) FCS and penicillin/streptomycin (Sigma-Aldrich). The expression plasmid of the enzymatically inactive mutant of pAPN (pAPNm) was prepared following Nam’s report (Nam & Lee, 2010) using pCAGGS-pAPN. The histidine residues in the enzymatic motif (HELH) were replaced with glutamine (QELAQ) by site-directed mutagenesis technique. CPK cells stably expressing pAPNm were prepared as described above.

**Viruses.** The PEDV MK strain (Shirato et al., 2010) was kindly provided by Dr Tetsuo Nunoya (Nippon Institute for Biological Science, Tokyo, Japan) and was maintained and titrated in Vero cells, as reported previously (Shirato et al., 2011). To prepare viral stocks under trypsin-free conditions, Vero cells were dispersed using cell dissociation solution (non-enzymatic) (Sigma-Aldrich) and seeded in culture dishes. The cells were inoculated with stock PEDV virus, which was allowed to adsorb to the cells for 60 min, and the cells were then washed twice and incubated in DMEM containing 10% (v/v) tryptose phosphate broth (TPB) liquid (10% TPB-DMEM) (Becton Dickinson) at 37°C. After 3 days of incubation, the cells and supernatants were pooled and the suspensions were ultrasonicated using a Biorupter UCD-250 (Tosho Electronics); the sonicated suspensions were used directly as virus stock suspensions. TGEV strain TO-163 was kindly provided by the National Institute of Animal Health, Ibaraki, Japan, and was propagated and titrated on CPK cells using standard TCID50 assays.

**Viral infection of pAPN-expressing cells.** To investigate viral growth kinetics in HeLa cells expressing pAPN, HeLa-pAPN,
Vero and CPK cells were inoculated with trypsin-free PEDV or TGEV at an m.o.i. of 0.01. After 60 min of adsorption, the cells were washed twice in PBS and incubated in 10% TBP-DMEM or 10% TBP-DMEM with 1.25 µg trypsin ml⁻¹ at 37°C. Cells and supernatants were separately collected at the indicated times after infection (0, 6, 18, 24 and 48 h) and virus titres were determined. To explore the effect of pAPN expression in CPK cells, PEDV and TGEV were inoculated into cells (CPK, CPK-pAPN and Vero cells) at an m.o.i. of 0.1. After 60 min of viral adsorption, the cells were washed twice with PBS and cultured in 10% TBP-DMEM or 10% TBP-DMEM with 1.25 µg trypsin ml⁻¹ at 37°C. After 3 days of infection, cells and supernatants were collected, and viral titres were determined.

For immunofluorescence assays, cells were fixed with methanol/acetone at 2 days post-infection with PEDV or TGEV. To detect PEDV and TGEV via immunofluorescence, sera from rabbits immunized with the NK94 strain of PEDV or the TO-163 strain of TGEV were used for the first staining. Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (Molecular Probes) was used as the secondary antibody. The cells were also counter-stained with DAPI. PEDV-inoculated Vero and HeLa cells were used as controls for staining. All images were captured on a Biozero BZ-8000 (Keyence).

**Real-time PCR assay.** Total RNAs were extracted using a total RNA isolation mini kit (Agilent). First-strand cDNA was synthesized with SMART MMLV reverse transcriptase (Clontech). As primers for reverse transcription, oligo-d(T)_18 and random hexamers (Applied Biosystems) were used for pAPN detection and random hexamers alone were used for the virus. Real-time PCR was performed using a LightCycler 480 system and Probe Master reagents (Roche) or a Fast Start SYBR Green master kit (Roche). For the detection of pAPN mRNA, a TaqMan gene expression assay (Applied Biosystems) was used. Viral RNA for the PEDV MK strain was detected using reported primer sets (forward 5'-ACGGCGACTACTCAGC-3' and reverse 5'-GGGCTATAAAGGGAATT-3') (Shirato et al., 2011). TGEV viral RNA was detected using reported primers (forward 5'-CCAGGGA TGGTGCATGAAC-3', reverse 5'-GGACTGTTGGCCTGCTCTAGA -3') (Yang et al., 2007). To evaluate viral cell entry, subgenomic mRNA for nucleocapsid (N) protein was detected using the following primers: for the PEDV MK strain, forward 5'-GGTCTGCTTCTCTGACTC-3', probe 5'-CCGGACACGGTGTG-3' and reverse 5'-GGGCA TAAAAGGGAATT-3'; for TGEV, forward 5'-GGTCTGCTCGACCAACTC-3' and reverse 5'-CCATAACACCTGTATTGAC-3'. The probe set for 18S rRNA (Applied Biosystems) was used as an internal control. Data were normalized to the value of the 18S RNA and calculated by a comparative method or a standard curve obtained using control human RNA (Applied Biosystems).

**Evaluation of PEDV production in HeLa cells.** To obtain concentrated viral RNA, Vero cells were infected with PEDV and cultured in the presence of 1.25 µg trypsin ml⁻¹. After 3 days of incubation, cells and supernatants were collected. One litre of virus culture was collected and centrifuged at 6500 g for 30 min at 4°C. The supernatants were collected and mixed with polyethylene glycol (PEG) and NaCl to a final concentration of 8% PEG 6000/0.5 M NaCl, and were incubated overnight at 4°C. After incubation, the mixtures were centrifuged at 6500 g for 30 min at 4°C and the pellets were dissolved in 10 ml PBS. The suspension was then subjected to ultracentrifugation at 110 000 g for 2 h at 4°C using a 20/60% (w/v) sucrose cushion. The interphase was collected and viral RNA was extracted using TRIzol LS reagent (Invitrogen). The copy number of the virus was calculated by means of real-time PCR assays, as described above. A total of 2 x 10⁸ copies of viral RNA were transfected into Vero or HeLa cells using DMRIE-C transfection reagent (Invitrogen), following the manufacturer's instructions. After 3 days of incubation, cells, and supernatants were collected together and subjected to ultrasonication using a Biorupter UCD-250 (Tosho Electronics), and then inoculated onto Vero cells. After the adsorption step, cells were washed with PBS twice and incubated in 10% TBP-DMEM containing 1.25 µg trypsin ml⁻¹. After 3 days of incubation, cells were fixed in methanol/acetic acid and stained as described above.

**pAPN activity assays.** In this study, leucine aminopeptidase (LAP) (microsomal, from porcine kidney) (L5006; Sigma-Aldrich) was used as a pAP. AP activity was evaluated as described by Chen et al. (2012). Briefly, APs were serially diluted in 100 µl volumes of 60 mM KH₂PO₄, pH 7.2. To evaluate AP activity, L-alanine-p-nitroanilide (Sigma-Aldrich) was used as the substrate and L-leucine-p-nitroanilide as the LAP control substrate. Equal amounts of pAP and substrate were mixed and incubated at 37°C for 60 min. Production of p-nitroanilide was measured using a microplate reader at 405 nm. L5006 is designated 'LAP', because the enzymatic activity is determined using leucine-containing substrates. However, L5006 is derived from porcine microsomes, and this product also contains APN, because an alanine residue could be released by L5006 from L-alanine-4-nitroanilide in a (enzyme) concentration-dependent manner (Fig. S1a, available in the online Supplementary Material). AP activity was also inhibited by bestatin, a general AP inhibitor (of both LAP and pAP) (Fig. S1b, c). These results suggest that L5006 contains pAPN and can be used as a source of soluble pAPN. Therefore, L5006 was used as a pAP in the following experiments.

To evaluate their AP activity, cells were cultured in 96-well plates. When the cells reached confluence, they were washed twice with PBS and directly used for APN activity assays, as described above (alanine substrate). Serially diluted pAPs were measured at the same time, the standard curve was calculated for each experiment, and the activity was expressed as ng APN ml⁻¹. To evaluate the role of pAPN as an enzyme, bestatin hydrochloride (Sigma-Aldrich) was used as a pAPN inhibitor. Cells were incubated in 10% DMEM containing 100 µM bestatin for 60 min. After treatment, cells were infected with viruses in the presence of bestatin for 60 min. After viral adsorption, cells were washed with PBS twice and incubated in 10% TBP-DMEM containing bestatin and 1.25 µg trypsin ml⁻¹ (for PEDV) or in 10% TBP-DMEM containing bestatin (for TGEV). After 3 days of incubation, cells and supernatants were collected together, and the viral titres were determined by plaque assay (for PEDV) or TCID₅₀ assay (for TGEV). Cell viabilities in the presence of bestatin were determined by staining with 0.2% (w/v) trypan blue/PBS at 0, 1, 2 and 3 days after treatment.

**pAPN-binding assay.** Hydrophobic magnetic Dynabeads M-270 epoxy beads were prepared following the manufacturer’s instructions. A total of 3.3 x 10⁸ beads were mixed with 100 µg LAP (Sigma-Aldrich) and incubated in 1 M ammonium sulfate at 37°C overnight. The beads were washed four times with PBS containing 0.5% (w/v) BSA and blocked with Immunoblock (DS Pharma Biomedical) for 1 h at room temperature. Binding of LAP to beads was confirmed via WB. Beads were boiled in SDS sample buffer for 30 min and dissolved proteins were examined via SDS-PAGE. After blotting to nylon membranes, LAP was detected using serum from LAP-immunized rabbits (from the National Institute of Animal Health) and peroxidase-conjugated, affinity-purified, anti-rabbit IgG guinea-pig antibody (Rockland). An ECL Prime Western blotting detection system (GE Healthcare) and the LAS-3000 imaging system (Fujifilm) were used to this end. The pAPN expression plasmid was transfected to 293T/17 cells and cell lysates were collected by CeLytic M cell lysis reagent (Sigma) in the presence of protease inhibitors (Roche) after 3 days of incubation. Cell lysates of CPK cells and control 293T/17 cells were also extracted. The size of pAPN was confirmed by WB analysis using L5006 and the above cell lysate, as described already. After blocking the beads, approximately 2.5 x 10⁶ copies of PEDV or TGEV in 100 µl and the same amount of Immunoblock were added to the beads, which were incubated for 2–3 h at 37°C. Next, the beads were washed four times with PBS and the level of viral RNA in the bound virions was measured via real-time PCR, as described above.

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Neutralization of PEDV by the soluble form of pAPN. To explore the neutralization activity of soluble pAPN against PEDV and TGEV, viruses were pretreated with pAPNs at the indicated concentrations (0, 37, 370, 37,000 ng ml⁻¹) at 37°C for 60 min. After neutralization of the viruses, Vero or CPK cells were inoculated with viruses and incubated for 60 min. After virus adsorption, cells were washed with PBS twice and incubated in 10% TPB-DMEM containing 1.25 µg trypsin ml⁻¹ (for PEDV) or in 10% TPB-DMEM containing pAPNs (for TGEV). After 24 hrs, total RNA was extracted from cells using a total RNA isolation mini kit (Agilent), and viral RNA was detected using the real-time PCR assay described above.

Receptor competition experiment. To assess pAPN usage as a receptor, competitive infection assays were performed between PEDV and TGEV. A total of 1×10⁵ Vero or CPK cells were seeded. Next, 1×10⁵ PEDV or TGEV were mixed with 1×10⁵ live or UV-inactivated competitor virus, after which they were inoculated onto cells. After 60 min of adsorption, cells were washed twice with PBS and incubated in 10% TPB-DMEM containing 1.25 µg trypsin ml⁻¹. After 24 hrs, cells were collected and detected for viruses using real-time PCR assays, as described above.

Statistical analysis. Statistical significance was explored with the aid of the unpaired t-test. A P value <0.05 was considered to indicate significance.

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