Porcine Deltacoronavirus Engages the Transmissible Gastroenteritis Virus Functional Receptor Porcine Aminopeptidase N for Infectious Cellular Entry

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ABSTRACT Identification of cellular receptors used by coronavirus (CoV) entry into the host cells is critical to an understanding of pathogenesis and to development of intervention strategies. The fourth CoV genus, *Deltacoronavirus*, evolutionarily related to the *Gammacoronavirus*, has just been defined recently. In the current study, we demonstrate that porcine aminopeptidase N (pAPN) acts as a cross-genus CoV functional receptor for both enteropathogenic porcine deltacoronavirus (PDCoV) and alphacoronavirus (AlphaCoV) (transmissible gastroenteritis virus [TGEV]) based upon three lines of evidence. First, the soluble S1 protein of PDCoV bound to the surface of target porcine cell lines known to express pAPN as efficiently as TGEV-S1, which could be blocked by soluble pAPN pretreatment. Second, both PDCoV-S1 and TGEV-S1 physically recognized and interacted with pAPN by coimmunoprecipitation in pAPN cDNA-transfected cells and by dot blot hybridization assay. Finally, exogenous expression of pAPN in refractory cells conferred susceptibility to PDCoV-S1 binding and to PDCoV entry and productive infection. PDCoV-S1 appeared to have a lower pAPN-binding affinity and likely consequent lower infection efficiency in pAPN-expressing refractory cells than TGEV-S1, suggesting that there may be differences between these two viruses in the virus-binding regions in pAPN. This study paves the way for dissecting the molecular mechanisms of PDCoV-host interactions and pathogenesis as well as facilitates future vaccine development and intervention strategies against PDCoV infection.

IMPORTANCE The emergence of new human and animal coronaviruses is believed to have occurred through interspecies transmission that is mainly mediated by a species-specific receptor of the host. Among the four genera of the *Coronavirinae*, a couple of functional receptors for the representative members in the genera *Alphacoronavirus* and *Betacoronavirus* have been identified, whereas receptors for *Gammacoronavirus* and *Deltacoronavirus*, which are believed to originate from birds, are still unknown. Porcine coronaviruses, including the newly discovered porcine deltacoronavirus (PDCoV) associated with diarrhea in newborn piglets, have posed a serious threat to the pork industry in Asia and North America. Here, we report that PDCoV employs the alphacoronavirus TGEV functional receptor porcine aminopeptidase N (pAPN) for cellular entry, demonstrating the usage of pAPN as a cross-genus CoV functional receptor. The identification of the PDCoV receptor provides another example of the expanded host range of CoV and paves the way for further investigation of PDCoV-host interaction and pathogenesis.

KEYWORDS cellular receptor, coronavirus, aminopeptidase N, APN, porcine deltacoronavirus, PDCoV, entry, virus entry

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Coronaviruses (CoVs) are single-stranded, positive-sense RNA viruses with the largest genome and cause mild or lethal respiratory and gastrointestinal diseases in humans and animals (1). Currently, the subfamily *Coronavirinae* of the family *Coronaviridae* is classified into four genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (1). The fourth genus, *Deltacoronavirus*, has just been defined recently (2). Since most of the gammacoronaviruses (GammaCoVs) and Delta-CoVs have been identified in avian species, birds are the proposed original host for these two genera, whereas bats are considered the original host for the genera *Alphacoronavirus* and *Betacoronavirus* (2).

Porcine deltacoronavirus (PDCoV), in particular, was isolated from pigs in the United States and many Asian countries, including China, and was found to cause severe diarrhea, vomiting, and dehydration in nursing piglets recently (3, 4). PDCoV and the other three emerging and reemerging swine enteric CoVs (SECoVs), including porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), and a newly discovered swine enteric alphacoronavirus (SeACoV) derived from the bat CoV-HKU2 (5), have caused a large number of pig deaths and significant economic impacts, which are considered a serious threat to the pork industry (3–7). PDCoV genomic RNA is approximately 25.4 kb in size. The genome organization of PDCoV is similar to that of the other reported coronaviruses, with the typical gene order of 5′-ORF1a/1b-spike (S)-envelope (E)-membrane (M)-NS6-nucleocapsid (N)/NS7-3′ (2, 8). PDCoV is closely related to the sparrow CoV-HKU17 (more than 90% amino acid identities in all seven domains in ORF1a/1b), and they are believed to be subspecies of the same species. Molecular clock analysis showed that the PDCoV jumped from birds to mammals approximately 523 years ago (2).

Identification of cellular receptors used by CoV for binding and entry into host cells is critical to understanding pathogenesis and to developing intervention strategies. To date, four types of CoV functional protein receptors have been identified: (i) aminopeptidase N (APN) for several AlphaCoVs including TGEV (9), (ii) carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), (iii) angiotensin-converting enzyme 2 (ACE2), and (iv) dipeptidyl peptidase 4 (DPP4) for three distinct BetaCoVs, namely, mouse hepatitis virus (MHV) (10), severe acute respiratory syndrome coronavirus (SARS-CoV) (11), and Middle East respiratory syndrome coronavirus (MERS-CoV) (12). Interestingly, the human ACE2 can also serve as the entry receptor for AlphaCoV human coronavirus (HCoV) NL63 in addition to SARS-CoV (13). These receptors interact with the amino-terminal receptor-binding domain S1 of specific CoV S glycoproteins, which determine the cross-species transmission and infection of CoVs (9–15).

While functional receptors for the representative members in *Alphacoronavirus* and *Betacoronavirus* genera have been continuously discovered, receptors for *Gammacoronavirus* and *Deltacoronavirus* members are still unknown. In the current study, we demonstrate that, similar to ACE2, porcine APN (pAPN) acts as a cross-genus CoV functional receptor for both porcine DeltaCoV (PDCoV) and AlphaCoV (TGEV) based upon three lines of evidence. First, a soluble S1-Fc fusion protein of PDCoV bound to the surface of target porcine cell lines known to express pAPN as efficiently as TGEV-S1-Fc, which could be blocked by soluble pAPN pretreatment. Second, both PDCoV-S1 and TGEV-S1 physically recognized and interacted with pAPN by coimmunoprecipitation (co-IP) in pAPN cDNA-transfected cells and by dot blot hybridization assay. Finally, exogenous expression of pAPN in refractory cells conferred susceptibility to PDCoV-S1 binding and, most importantly, for PDCoV entry and productive infection.

RESULTS

Soluble TGEV-S1 and PDCoV-S1 binding to porcine permissive cells endogenously expressing pAPN. It has been well known that swine testicular (ST) cells and porcine kidney epithelial LLC-PK1 cells are permissive for TGEV infection (9, 16). We noticed that PDCoV was initially isolated and propagated in these two cell lines (3, 4), suggesting a common cell tropism for TGEV and PDCoV. In addition, neither African green monkey Vero cells (ATCC CCL-81) nor hamster BHK-21 cells were permissive for

FIG 1 Soluble TGEV-S1 and PDCoV-S1 bind to porcine permissive cells endogenously expressing pAPN. (A) Binding of soluble TGEV S1 protein to the cell surface as determined by flow cytometry analysis. Equal amounts (10 μ g/ml) of TGEV-S1-hFc (filled histogram) or hFc (dashed line) were incubated with susceptible LLC-PK1 or ST cells or with nonsusceptible Vero or BHK-21 cells. Cell surface binding was detected by an FITC-conjugated anti-human IgG Fc. (B) Binding of soluble PDCoV S1 to LLC-PK1, ST, Vero, or BHK-21 cells. Equal amounts (10 μ g/ml) of PDCoV-S1-hFc (filled histogram) or Fc only (dashed line) were incubated with four cell lines, followed by FITC-conjugated anti-human IgG Fc detection. (C) Detection of endogenous expression of pAPN on LLC-PK1 or ST cells or of pAPN exogenous expression on Vero-pAPN or BHK-pAPN stable cells by immunoblot analysis using an anti-APN Ab. Max, maximum.

TGEV or PDCoV infection *in vitro* in our lab. To investigate whether PDCoV-S1 determines the cell tropism, as has been documented for TGEV-S1 (14), we generated S1-human Fc (hFc) chimeric proteins from PDCoV (Chinese/Hunan strain; GenBank accession no. [KY513724](#)) and TGEV (prototype Purdue strain) (5), respectively. As expected, soluble TGEV-S1-hFc bound to target LLC-PK1 and ST cells but not to nonsusceptible Vero or BHK-21 cells, as determined by flow cytometry analysis (Fig. 1A). Next, we tested the binding of soluble PDCoV-S1 under the same conditions. Comparison of the cell surface binding abilities of PDCoV-S1-hFc to LLC-PK1 and ST cells indicated significant similarities with TGEV-S1 binding, whereas S1-hFc binding was not detected in Vero or BHK-21 cells (Fig. 1B), which correlates with nonsusceptibility to infection by PDCoV.

We further confirmed that both LLC-PK1 and ST cells had endogenous expression of pAPN whereas Vero and BHK-21 cells lacked corresponding APN expression, as determined by Western blotting (WB) analysis using a broadly reactive anti-APN antibody (Ab) (Fig. 1C). As controls, two stable cell lines, Vero-pAPN and BHK-pAPN, both expressing pAPN, were established by transfection with a recombinant construct, pAPN-Myc, expressing full-length pAPN cDNA fused with a Myc tag at the C terminus, followed by selection with puromycin. Expression of pAPN was detected in Vero-pAPN cells (Fig. 1C). Comparison of APN expression levels between BHK-21 cells and BHK-pAPN cells had a similar result, showing pAPN expression only in BHK-pAPN cells (Fig. 1C). Thus, LLC-PK1 and ST cells are susceptible to both TGEV-S1 and PDCoV-S1 binding, permissive to both TGEV and PDCoV infection, and express the TGEV receptor pAPN, whereas Vero and BHK-21 cells are not susceptible to binding, not permissive to infection, and do not express APN.

FIG 2 PDCoV-S1 and TGEV-S1 interact with pAPN. (A) BHK-21 cells were transfected with equal amounts (2 μ g) of the empty vector pFUSE-Fc alone, pFUSE-Fc and pAPN-Myc (with a C-terminal Myc-tag), PDCoV-S1-Fc alone, PDCoV-S1-Fc and pAPN-Myc, TGEV-S1-Fc alone, and TGEV-S1-Fc and pAPN-Myc. The whole-cell lysate (WCL) was preadsorbed onto protein A-conjugated agarose beads prior to SDS-PAGE for co-IP analysis or was used for SDS-PAGE directly. The protein complex was detected by using an HRP-conjugated anti-Myc Ab and an HRP-conjugated anti-Fc Ab. (B) Detection of binding of human Fc-tagged protein (TGEV-S1-hFc, PDCoV-S1-hFc, or hFc) to soluble pAPN by dot blot hybridization assay. The porcine APN ectodomain tagged with a C-terminal murine IgG1 Fc (pAPN-mFc) or mFc was spotted on nitrocellulose membrane. Binding of S1-hFc proteins to pAPN was detected by using an HRP-conjugated anti-human Fc Ab.

Interaction between PDCoV-S1/TGEV-S1 and pAPN associated with cell tropism. The interaction between PDCoV-S1 and pAPN was analyzed by co-IP. BHK-21 cells were transfected with a PDCoV-S1-hFc or TGEV-S1-hFc expression construct alone or with an empty vector expressing hFc alone or cotransfected with each hFc construct with a pAPN-Myc plasmid. Expression of transfected Fc-tagged proteins and pAPN was confirmed in whole-cell lysates (WCLs) by WB with anti-Fc Ab and anti-Myc Ab, respectively (Fig. 2A, bottom). The transfected BHK-21 cells were then immunoprecipitated with Fc tag preadsorbed onto protein A-conjugated agarose beads. The bound protein complexes were subjected to WB analysis with anti-Myc Ab or anti-Fc Ab. As shown in Fig. 2A (top), pAPN-Myc bound specifically to both PDCoV-S1-hFc and TGEV-S1-hFc. In contrast, pAPN-Myc did not bind to the control hFc protein (Fig. 2A, lanes 3 and 5). Notably, the amount of pAPN-Myc brought down by PDCoV-S1-hFc (Fig. 2A, lane 4, top) was significantly less than that brought down by TGEV-S1-hFc (lane 6, top), whereas the expression level of pAPN-Myc is more abundant in lane 4 than in lane 6. Since equal amounts of plasmid DNA of PDCoV-S1-hFc and TGEV-S1-hFc served as

input for the IP experiment, as shown by the absence of significant differences in the detection levels between S1-Fc proteins either in IP or in WCLs (Fig. 2A), the IP result suggests that PDCoV-S1 may have a lower pAPN-binding affinity than TGEV-S1.

To further validate the specific interaction between PDCoV-S1 and pAPN, a dot blot hybridization assay was conducted. It was shown that both TGEV-S1-hFc and PDCoV-S1-hFc efficiently bound to the soluble pAPN ectodomain tagged with a mouse Fc (pAPN-mFc) but not to the mFc control. On the other hand, the hFc bound to neither pAPN-mFc nor mFc (Fig. 2B). These results demonstrated that both PDCoV-S1 and TGEV-S1 physically recognized and interacted with pAPN.

Next, the soluble pAPN-mFc or mFc was preincubated with TGEV-S1-hFc, PDCoV-S1-hFc, or hFc; the LLC-PK1 or ST cells were then subjected to flow cytometry analysis with the mixtures as described in the legends of Fig. 1A and B. Treatment of S1-hFc with pAPN-mFc but not mFc blocked surface binding (Fig. 3A), indicating that PDCoV-S1 and TGEV-S1 employ pAPN for cellular binding on host (swine) cells. These data collectively demonstrate a direct and specific interaction between PDCoV-S1/TGEV-S1 and pAPN associated with cell tropism.

Exogenous pAPN expression in refractory Vero or BHK-21 cells confers susceptibility to PDCoV-S1 and TGEV-S1 binding. We next determined whether pAPN could indeed mediate PDCoV entry in refractory cells. The full-length cDNA encoding pAPN fused with a Myc tag (pAPN-Myc) was stably expressed by puromycin selection in Vero or BHK-21 cell lines (Vero-pAPN or BHK-pAPN), as shown by WB analysis (Fig. 1C). Furthermore, surface expression of pAPN on Vero-pAPN or BHK-pAPN cells was validated by detection by flow cytometry of efficient binding of TGEV-S1-hFc and PDCoV-S1-hFc, which could be blocked by soluble pAPN-mFc pretreatment (Fig. 3B), similar to what was observed in LLC-PK1 and ST cells (Fig. 3A). In contrast, the two S1-hFc proteins did not bind to the parental cell lines (Fig. 3B), which was in line with the result shown in Fig. 1A and B. As controls, the two S1-hFc soluble proteins did not bind to Vero cells overexpressing the SARS-CoV and HCoV-NL63 receptor ACE2 (11, 13) or to BHK-21 cells exogenously expressing ACE2 (Fig. 3B). Therefore, exogenous expression of pAPN in refractory Vero or BHK-21 cells conferred specific susceptibility to PDCoV-S1 and TGEV-S1 binding. In addition, cytoplasmic expression of pAPN in Vero-pAPN or BHK-pAPN cells was also validated by immunofluorescence assay (IFA) using anti-Myc Ab or anti-pAPN Ab (Fig. 4A).

Exogenous pAPN expression allows refractory cell lines to support PDCoV efficient replication and productive infection. The Vero, Vero-pAPN, BHK-21, and BHK-pAPN cell lines were inoculated with either TGEV or PDCoV at a multiplicity of infection (MOI) of 0.1. As expected, TGEV N protein antigens were detected and spread in the cytoplasm of 35 to 40% of both Vero-pAPN and BHK-pAPN cells by 36 h postinoculation (hpi), but no viral antigens were found in challenged Vero or BHK-21 cells (Fig. 4A, top). PDCoV also infected Vero-pAPN cells with an efficiency of 25 to 30% and BHK-pAPN cells with an efficiency of 30 to 35% but did not infect Vero or BHK-21 cells by 36 hpi, as assessed by detection of specific PDCoV N protein expression (Fig. 4A, bottom). A slightly lower infection efficiency of PDCoV than that of TGEV could likely be accounted for by the possibly lower pAPN-binding affinity of PDCoV-S1 (Fig. 2A). Development of cytopathic effects (CPEs) characterized by cell rounding, aggregation, and subsequent detachment in PDCoV-infected Vero-pAPN cells was observed (Fig. 4B).

To determine whether pAPN-expressing cells could confer PDCoV replication competency, Vero-pAPN or PDCoV target LLC-PK1 cells were inoculated with PDCoV (MOI of 0.1) with or without soluble pAPN preincubation, and the viral RNA levels in the supernatant of cell lysates at 2, 8, and 24 hpi were assessed by quantitative reverse transcription-PCR (qRT-PCR). Figure 5 showed that PDCoV RNA was synthesized gradually from two types of cells. Moreover, soluble pAPN preincubation with PDCoV blocked viral replication at the early stage (2 and 8 hpi), indicating that replication-competent PDCoV utilizes pAPN as an entry receptor (Fig. 5). At 24 hpi, inhibition of PDCoV replication by soluble pAPN was not significant in either cell line, suggesting

FIG 3 Flow cytometry analysis of cell surface expression of pAPN. (A) Soluble pAPN-mFc (10 μ g/ml) preincubation was able to block TGEV-S1-hFc and PDCoV-S1-hFc binding to permissive LLC-PK1 and ST cells (gray-filled histogram), whereas preincubation with soluble mFc (10 μ g/ml) did not block TGEV-S1-hFc or PDCoV-S1-hFc binding (black-filled histogram). Preincubation with pAPN-mFc followed by hFc binding was used as the control (dashed line). Cell surface binding was detected by an FITC-conjugated anti-human IgG Fc. (B) TGEV-S1-hFc and PDCoV-S1-hFc bound to BHK-21 or Vero cells stably expressing pAPN (BHK-pAPN and Vero-pAPN, respectively; red histograms) but not to BHK-21 or Vero (green histograms) or to BHK-21 or Vero cells expressing ACE2 cells (blue histograms). Soluble pAPN-mFc (10 μ g/ml) preincubation was able to block TGEV-S1-hFc and PDCoV-S1-hFc binding to BHK-pAPN and Vero-pAPN cells (yellow histogram). Cell surface binding was detected as described in the legend of Fig. 1A and B.

FIG 4 Vero or BHK-21 cells stably expressing pAPN confer susceptibility to PDCoV and TGEV infection. (A) Vero, Vero-pAPN, BHK-21, and BHK-pAPN cells were challenged with TGEV or PDCoV. At 36 h postchallenge, TGEV-challenged cells were costained with a rabbit anti-TGEV-N pAb and a mouse anti-Myc MAb, whereas PDCoV-challenged cells were costained with a mouse anti-PDCoV-N MAb and a rabbit anti-APN pAb. Alexa Fluor 488- or 594-conjugated anti-rabbit or anti-mouse IgG was costained for secondary (Continued on next page)

FIG 5 Infection of PDCoV in LLC-PK1 or Vero-pAPN cells was inhibited by soluble pAPN (at a concentration of 39 or 78 µg/ml) within 24 h. PDCoV RNA titers were measured by one-step quantitative RT-PCR targeting the M gene. *, $P < 0.05$; **, $P < 0.01$. GE, genome equivalents.

that PDCoV is probably propagated and spread from cell to cell by this time point (Fig. 5).

The progressive PDCoV release into the cultured medium (extracellular) was determined by dynamic viral RNA synthesis and virus titers. Both extracellular and intracellular PDCoV RNA and extracellular virus titers could be detected in the supernatants of Vero-pAPN and the control LLC-PK1 cells but not in the supernatants of Vero cells during a period of 72 hpi (Fig. 6A and B), indicating RNA replication and a productive PDCoV infection of Vero-pAPN cells. Since the extracellular PDCoV infectious titers were assessed on fresh LLC-PK1 cells by endpoint dilutions (titration), the result also demonstrated that PDCoV secreted from Vero-pAPN cells could be passaged (Fig. 6B). Progeny PDCoV infection of fresh LLC-PK1 cells was also validated by IFA using an anti-PDCoV-N Ab (Fig. 6C). The PDCoV growth curve in Vero-pAPN cells was lower than that in control LLC-PK1 cells but reached peak titers in the period of 48 to 72 hpi ($5.12 \log_{10} 50\%$ tissue culture infective doses [TCID₅₀]/ml and $6.27 \log_{10} \text{TCID}_{50}/\text{ml}$, respectively, for Vero-pAPN and LLC-PK1 cells).

For comparison of infection efficiencies, we also determined the growth kinetics of TGEV secreted from Vero-pAPN, Vero, or control LLC-PK1 cells on fresh LLC-PK1 cells (Fig. 6B). No TGEV was produced in Vero cells. In control LLC-PK1 cells, extracellular TGEV had a growth curve with virus titers analogous to those of extracellular PDCoV, while for Vero-pAPN cells inoculated with either TGEV or PDCoV, extracellular TGEV propagated more efficiently than extracellular PDCoV, reaching a peak titer at 48 hpi ($5.67 \log_{10} \text{TCID}_{50}/\text{ml}$) and with approximately 3- to 10-fold higher titers during 6 to 48 hpi (Fig. 6B). The kinetics data were consistent with the distinct expression levels of N proteins between TGEV and PDCoV (Fig. 4A).

These results collectively demonstrated that exogenous expression of recombinant pAPN in refractory cell lines is sufficient to allow binding, entry, synthesis of viral RNA

FIG 4 Legend (Continued)

antibody detection, followed by DAPI incubation. Magnification, $\times 200\times$. (B) Evidence of PDCoV infection in Vero-pAPN cells showing cytopathic effects (CPEs) with cell rounding and aggregation (indicated by red arrows) at 36 h postinfection.

FIG 6 Determination of the kinetics of PDCoV replication, propagation, and release in Vero-pAPN or control LLC-PK1 cells. (A) The amounts of extracellular and intracellular viral RNA in Vero-pAPN, Vero, and LLC-PK1 cells were assessed in triplicate by qRT-PCR, respectively. (B) Virus titers of PDCoV or TGEV released into the supernatant of inoculated Vero-pAPN, Vero, or LLC-PK1 cells were determined in triplicate on fresh LLC-PK1 cells. Samples of supernatants and cells were collected at intervals between 2 and 72 h postinoculation. Error bars indicate standard deviations. (C) Infection of fresh LLC-PK1 cells with progeny PDCoV collected from Vero-pAPN cells inoculated with PDCoV. IFA was performed at 36 hpi. The expression of PDCoV N protein was detected by staining with anti-PDCoV-N MAb and Alexa Fluor 488-conjugated goat anti-mouse IgG Ab (middle panel).

and protein, and release of infectious PDCoV. Therefore, pAPN serves as a functional receptor for both PDCoV and TGEV.

DISCUSSION

Identification of the host functional receptor for a pathogenic virus is very important for understanding the mechanisms of virus-host interplay. APN, also known as a type II zinc metalloprotease, mediates the entry of most of AlphaCoVs (9, 17, 18). Our study indicated that PDCoV in the newly defined *Deltacoronavirus* genus engages the same pAPN receptor, which is expressed in abundance in the porcine small intestinal mucosa, to infect the same target cells as TGEV (9), likely leading to induction of clinical signs of diarrhea in piglets. The highly conserved feature of receptor engagement between PDCoV and TGEV is consistent with a closely molecular architecture of the S glycoproteins (19), common cell tropism (4, 9, 16), and consequent pathogenesis exhibited by these two SECoVs in distinct CoV genera (3).

Intriguingly, among four pathogenic SECoVs, the AlphaCoV PEDV was previously reported to utilize pAPN as an entry receptor by using a pseudotype assay with PEDV S protein (20). However, recent studies have demonstrated that pAPN, human APN (hAPN), and green monkey APN are not functional receptors for PEDV in cultured porcine, human, or Vero cells (21–23). Moreover, we found that a recombinant PEDV expressing green fluorescent protein (GFP), PEDV-GFP (23) and other Chinese and U.S. genogroup-1 and genogroup-2 PEDV strains (6, 24, 25) did not infect BHK-21 or BHK-pAPN cells (data not shown). Although BHK-21 is a cell line refractory to infection with PEDV, TGEV, and PDCoV, it can support PEDV replication and production by transfection of PEDV infectious cDNA clones (unpublished data). The comparative results reveal that BHK-pAPN cells were susceptible to TGEV and PDCoV but not PEDV infection, providing additional evidence of the exclusive engagement of pAPN with PDCoV and TGEV.

Most recently, our lab discovered a novel bat CoV-HKU2-related swine enteric virus, SeACoV, in southern China (5). The SeACoV is unique since it has an AlphaCoV genomic backbone with an S gene phylogenetically related to that from BetaCoV (5). Members of the BetaCoVs have not been found to use APN as a receptor, and SeACoV, similar to PEDV, infects monkey APN-deficient Vero cells (5), suggesting that APN is likely not the entry receptor for SeACoV.

TGEV infection is highly naturally host specific *in vitro* and *in vivo* in that TGEV uses pAPN but not hAPN as its cellular receptor. A related human AlphaCoV, HCoV-229E, utilizes hAPN but not pAPN to enter host cells (17, 26). Mutagenesis studies and subsequent determination of the crystal structure of pAPN revealed that different virus-binding motifs (VBMs) in pAPN/hAPN containing a species-specific N-linked glycan are required to mediate susceptibility to infection with TGEV and HCoV-229E (27, 28). TGEV recognizes APN residues 728 to 744 (VBM2 region) whereas HCoV-229E recognizes residues 283 to 292 (VBM1 region); both regions are located on the outer surface of APN and can be approached easily by viruses (27). Furthermore, the crystal structure of the porcine respiratory CoV (PRCV; a TGEV variant) receptor-binding domain (RBD) on S1 in complex with pAPN revealed that the Tyr-304 and Trp-347 residues at the two RBD protruding tips penetrate small cavities of the APN domain DIV (containing VBM2) and that the other RBD residues contact an N-acetylglucosamine linked to Asn-736 of pAPN, which is critical for PRCV/TGEV binding to APN (29). The absence of an APN-binding Tyr and a different conformation of the receptor-binding loop at the tip in HCoV-229E RBD are in line with a distinct VBM (e.g., VBM1) recognized by HCoV-229E although the structure of the complex of HCoV-229E RBD and hAPN has not yet been resolved. More recently, a nearly atomic-level resolution of the structure of the PDCoV S glycoprotein trimer by cryo-electron microscopy has revealed that the PDCoV RBD displays a β -sandwich fold reminiscent of that of AlphaCoVs, harboring topologically similar glycosylation sites on the β -sandwich surface (19). Several aromatic residues (Phe-318, Tyr-394, Trp-396, and Tyr-398) at the protruding tips have also been speculated to mediate a receptor-RBD interaction (19).

However, since PDCoV can also infect some human cell lines, such as Huh-7 expressing hAPN *in vitro* (preliminary data not shown) and calves *in vivo* (30) and could be detected in Asian leopard cats and Chinese ferret badgers (31), we hypothesize that PDCoV can utilize APNs from other mammalian species as receptors, which distinguishes TGEV and HCoV-229E in usage of host-specific APN. If so, the VBM in APN recognized by PDCoV-S1 may be different from the VBM1 of HCoV-229E or VBM2 recognized by TGEV. This hypothesis is also supported by the experimental data from this study, where we found that the results of co-IP, IFA, and comparative growth kinetics (Fig. 2A, 4A, and 6B) indicated the likely differences in receptor-binding affinities and in consequent infection efficiencies in Vero-pAPN cells between PDCoV and TGEV. If so, the structural basis for this APN binding by cross-genus CoVs is totally distinct from what has been known for ACE2 binding by SARS-CoV and HCoV-NL63 (32, 33). SARS-CoV does not have a structure in the RBD core that is homologous to that of HCoV-NL63, but both viruses recognize a virus-binding “hot spot” in the common ACE2 region (32). Future studies on mapping of the PDCoV-binding VBM in pAPN and resolving the structure of PDCoV-RBD in complex with pAPN are warranted to confirm our hypothesis.

Since *Deltacoronavirus* is believed to originate from avian CoVs (2), our result also raises a question as to whether the APN counterparts in avian species play a role in PDCoV cross-species transmission. Nevertheless, the present study suggests that PDCoV has evolved a mechanism that utilizes pAPN as its entry receptor to expand the host range. The identification of pAPN as a functional receptor used by PDCoV paves the way for dissecting the molecular mechanisms of PDCoV-host interactions and pathogenesis as well as facilitating future vaccine development and intervention strategies against PDCoV infection.

MATERIALS AND METHODS

Cell lines, virus stocks, and viral antibodies. A porcine kidney epithelial cell line, LLC-PK1 (ATCC CL-101), a swine testis cell line, ST (ATCC CRL-1746), a baby hamster kidney fibroblast cell line, BHK-21 (ATCC CCL-10), and an African green monkey kidney epithelial Vero cell line (ATCC CCL-81) were individually grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% (wt/vol) antibiotics (penicillin and streptomycin). All cells were grown at 37°C with 5% CO₂.

The TGEV Purdue strain (a gift from Rong Ye at Shanghai Medical College of Fudan University, China) was produced in ST cells, whereas the PDCoV CH/Hunan/2014 strain (GenBank accession no. [KY513724](#)) was propagated in LLC-PK1 cells (5). ST or LLC-PK1 cells were seeded at 70% confluence in T-25 flasks and incubated overnight. After the cells were washed with phosphate-buffered saline (PBS), viruses at an MOI of 0.1 were added to each flask. After 2 h of incubation at 37°C, the cells were washed with PBS and then cultured in DMEM without FBS. Cells were observed daily to check for any cytopathic effect (CPE). When CPE was obvious, whole-cell cultures (supernatant and cells) were harvested and subjected to three freeze-thaw cycles prior to removal of cell debris by centrifugation. The virus titers of TGEV or PDCoV were determined by endpoint dilutions as the 50% tissue culture infective doses (TCID₅₀) on fresh cells as described previously (24). Virus stocks were stored at −80°C until use.

Anti-PDCoV-nucleocapsid (N) monoclonal antibody (MAb) was purchased from Medgene Labs (Brookings, SD, USA), whereas anti-TGEV-N polyclonal antibody (pAb) was generated in-house. Briefly, the full-length N protein of TGEV with a six-histidine tag expressed in *Escherichia coli* was purified and used to immunize two New Zealand White rabbits. Antiserum was harvested and affinity purified at 55 days postimmunization.

Construction of the recombinant plasmids. The complete coding region of porcine APN (pAPN; GenBank accession no. [KX342854](#)) was amplified by one-step RT-PCR using total RNAs extracted from porcine small intestine and subsequently cloned into a pCI-neo vector (Promega, USA) using NheI and SalI restriction sites. The recombinant plasmid was designated pCI-pAPN (23). Expression of pAPN protein *in vitro* was confirmed by transient transfection of pCI-pAPN in BHK-21 or Vero cells by immunofluorescence assay (IFA) with an anti-APN pAb (93897; Abcam) (23). Next, the pAPN ectodomain encoding amino acids (aa) 62 to 963 (27) was amplified from pCI-pAPN and inserted into a pFUSE-mlgG1-Fc2 vector (Invivogen, USA) containing an in-frame C-terminal murine IgG1 Fc (mFc) fragment to construct an expression construct named pAPN-mFc. The third pAPN-expressing construct, pAPN-Myc, harboring the full-length pAPN cDNA fused with a Myc tag at the C terminus, was engineered between XhoI and HpaI restriction sites in the lentiviral vector pLV-CMV-PURO (kindly provided by Pinglong Xu at the Life Sciences Institute of Zhejiang University). For construction of soluble human IgG1 Fc (hFc) fusion proteins, TGEV-S1 (aa 1 to 832) and PDCoV-S1 (aa 1 to 574) subunits were amplified by PCR from the respective full-length cDNA of the viral genome and inserted into a pFUSE-hlgG1-Fc1 vector (Invivogen, USA). The recombinant plasmids were designated pFUSE-TGEV-S1-hFc and pFUSE-PDCoV-S1-hFc. The

expression plasmid harboring the full-length cDNA of the SARS-CoV cellular receptor ACE2, pCMV3-Flag-ACE2 (HG10108-NF), was purchased from Sino Biological, Inc. (Beijing, China).

Generation of two stable cell lines expressing pAPN. Two stable cell lines, Vero-pAPN and BHK-pAPN, were established in Vero and BHK-21 cells by transfection with the construct pAPN-Myc followed by selection with 10 μ g/ml of puromycin, as described previously (34). Expression of the pAPN-Myc fusion protein was confirmed by IFA with an anti-Myc MAb (2276; Cell Signaling Technology) and an anti-APN pAb. PDCoV or TGEV was inoculated and cultured in Vero-pAPN, Vero, BHK-pAPN, or BHK-21 cells in the presence of trypsin (3 μ g/ml; Sigma).

Flow cytometry analysis. We followed a protocol as described previously by Li et al. (21). Briefly, soluble hFc and mFc fusion proteins (TGEV-S1-hFc, PDCoV-S1-hFc, hFc, mFc, and pAPN-mFc) were transiently expressed in 293T cells and affinity purified from the supernatant medium using protein A-Sepharose beads (Transbionovo, Beijing, China). Analysis of purified proteins was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to ensure the purity and quality (data not shown). Binding of soluble Fc proteins to the surface of given cells was performed by incubation with 10 μ g/ml of purified S1-hFc proteins followed by detection with a fluorescein isothiocyanate (FITC)-conjugated anti-human IgG Fc Ab (Thermo Fisher Scientific) by flow cytometry. Blocking of S1-hFc was performed by preincubation with soluble pAPN-mFc or mFc for 2 h prior to performance of a surface binding assay.

Immunofluorescence assay, Western blotting, and dot blot hybridization assay. Plasmids were transiently transfected into Vero or BHK-21 cells using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's protocol. Transfected cells or virus-infected cells were cultured for 36 to 72 h and then subjected to immunofluorescence assay, co-IP, or Western blotting to detect protein expression or interaction. For IFA, cells were washed twice with phosphate-buffered saline (PBS) and fixed with acetone. Cells were then incubated with the primary Ab. After incubation for 1 h at room temperature, the cells were washed with PBS and stained with the secondary Ab followed by 4',6-diamidino-2-phenylindole (DAPI) staining. For Western blot analysis, cells were lysed in 125 μ l of Cellytic M lysis buffer (Sigma) per 10⁶ cells. The whole-cell lysates (WCLs) were preadsorbed onto protein A-conjugated agarose beads prior to SDS-PAGE for co-IP analysis or used for SDS-PAGE directly. Samples were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane that was subsequently blocked with Tris-buffered saline (TBS) containing 3% bovine serum albumin (BSA) overnight at 4°C. Proteins were detected using the primary Ab followed by incubation with horseradish peroxidase (HRP)-conjugated secondary Ab (Thermo Fisher Scientific). Binding of Fc-tagged proteins (TGEV-S1-hFc, PDCoV-S1-hFc, and hFc) to soluble pAPN-mFc or mFc was detected by dot blot hybridization assay as described by Li et al. (21).

Quantitative RT-PCR. Total RNA was extracted from supernatant medium and cell lysates of PDCoV-inoculated cells at different time points using an AxyPrep Multisource Total RNA Miniprep kit (Axygen). PDCoV RNA titer was monitored by one-step qRT-PCR targeting the membrane (M) gene with the primers 5'-ATCGACCACATGGCTCCAA-3' and 5'-CAGCTCTTGCCCATGTAGCTT-3' and the probe FAM-CACACAGTCGTTAAGCATGGCAAGCT-BHQ (where FAM is 6-carboxyfluorescein and BHQ is Black Hole quencher) as described previously (35, 36). Standard curves were performed to allow absolute quantitation of PDCoV RNA copy numbers based upon the levels of *in vitro*-transcribed RNA containing the targeting sequences. Samples with a cycle threshold value of <35 were considered positive based upon validation data using the standard RNA.

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