Broad receptor engagement of an emerging global coronavirus may potentiate its diverse cross-species transmissibility

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Coronaviruses (CoVs) are enveloped positive-strand RNA viruses—classified into four genera: Alpha-, Beta-, Gamma-, and Deltacoronavirus (subfamily Coronavirinae, family Coronaviridae)—that exhibit a propensity for interspecies transmission (1, 2). The betacoronaviruses severe acute respiratory syndrome (SARS)-CoV and Middle East respiratory syndrome (MERS)-CoV, both of which can cause lethal respiratory infections in humans, are notable examples of CoVs crossing species barriers. SARS-CoV became human-adapted after a zoonotic introduction in 2002 and quickly spread to infect thousands worldwide before its containment in 2003 (3, 4). MERS-CoV, discovered a decade later, has not adapted to sustained replication in humans as yet, but causes recurrent spillover infections from its reservoir host, the dromedary camel (5). Phylogenetic studies indicate that cross-species transmission has occurred relatively frequently during CoV evolution and shaped the diversification of CoVs (6). In fact, the endemic human coronaviruses HCoV-HKU1, HCoV-229E, HCoV-NL63, and HCoV-OC43 all have a zoonotic origin (7–11). Occurrence of such cross-species transmission events may be attributed to widespread CoV prevalence in mammals and birds, and to their extraordinary variability stemming from high mutation rates and high frequency recombination, which greatly increase the potential for successful adaptation to a new host (6, 12). A pivotal criterion of cross-species transmission concerns the ability of a virus to engage a receptor within the novel host, which for CoVs, is determined by the receptor specificity of the viral spike (S) protein.

The porcine deltacoronavirus (PDCoV) is a recently discovered CoV of unknown origin. PDCoV (species name coronavirus HKU15) was identified in Hong Kong in pigs in the late 2000s (13) and has since been detected in swine populations in various countries worldwide (14–24). It infects the intestinal epithelia and can cause acute, watery diarrhea and vomiting, resulting in dehydration and body weight loss with potentially fatal consequences (23, 25, 26). So far, all other members of the Deltacoronavirus genus have been detected in birds, suggesting that birds are the natural host and ancestral reservoir of deltacoronaviruses (13). PDCoV is most closely related to the sparrow CoV HKU17. Pairwise genome analysis shows that these two viruses are subspecies of the same species with >96% amino acid identity in domains used for species demarcation (13, 27), indicating that an interspecies transmission event from birds to mammals is likely.

PDCoV | spike | APN | receptor | cross-species transmission

Coronaviruses exhibit a propensity for interspecies transmission, with SARS- and MERS-coronaviruses as notable examples. Cross-species transmission by coronaviruses is foremost determined by the virus’ ability to bind receptors of new hosts. We here report that the recently identified, yet globally distributed porcine deltacoronavirus employs aaminopeptidase N (APN) as an entry receptor, which for CoVs, is determined by the receptor specificity of the viral spike (S) protein. Here, we report that PDCoV employs host aminopeptidase N (APN) as an entry receptor and interacts with APN via domain B of its spike (S) protein. Infection of porcine cells with PDCoV was drastically reduced by APN knockout and rescued after reconstitution of APN expression. In addition, we observed that PDCoV efficiently infects cells of unusual broad species range, including human and chicken. Accordingly, PDCoV S was found to target the phylogenetically conserved catalytic domain of APN. Moreover, transient expression of porcine, feline, human, and chicken APN renders cells susceptible to PDCoV infection. Binding of PDCoV to an interspecies conserved site on APN may facilitate direct transmission of PDCoV to nonreservoir species, including humans, potentially reflecting the mechanism that enabled a virus, ancestral to PDCoV, to breach the species barrier between birds and mammals. The APN cell surface protein is also used by several members of the Alphacoronavirus genus. Hence, our data constitute the second identification of CoVs from different genera that use the same receptor, implying that CoV receptor selection is subjected to specific restrictions that are still poorly understood.
mammals may have occurred relatively recently. Interestingly, the S proteins of the bulbous CoV HKU11 and mumps CoV HKU13 show higher sequence identity with the PDCoV S protein compared with that of HKU17 (70.2% and 71.2% vs. 44.8%), suggesting that a recombination event predated emergence of this porcine CoV (13).

Studying PDCoV spike–receptor interactions may provide insight into the presumed host-switching event from birds to swine. The CoV S protein forms homotrimers and is composed of an N-terminal S1 subunit and a C-terminal S2 subunit, responsible for receptor binding and membrane fusion, respectively. Recent cryo-EM reconstructions of the CoV trimeric S structures of alpha-, beta-, and deltacoronaviruses (28–32) revealed that the S1 subunit comprises four core domains (S1A–D), of which domains A and B have been implicated in receptor binding. So far, a surprisingly limited set of four cell surface host glycoproteins have been reported to be used as receptors by CoVs. The carcinoembryonic antigen-related cell-adhesion molecule 1 is recognized as a receptor by the lineage A betacoronavirus MHV (33). The three remaining receptors are all membrane ectopeptidases, one of which is used by members from different genera. The aminopeptidase N (APN) is targeted by a number of alphacoronaviruses, including HCoV-229E and transmissible gastroenteritis virus (TGEV) (34, 35). Dipeptidyl peptidase 4 (DPP4) was shown to be used as a receptor by the lineage C betacoronavirus MERS-CoV (36). Finally, the peptidase angiotensin converting enzymes 2 (ACE2) is used as a receptor by the alphacoronaviruses HCoV-NL63, as well as by the (lineage B) betacoronavirus SARS-CoV (37, 38). In addition to proteinaceous host molecules, (acyetylated) sialic acid carbohydrates may be used as primary receptors or as attachment factors (39–42). The entry receptor for PDCoV is unknown, as well as for any of the other deltacoronaviruses identified thus far. In this study, we aimed to identify and characterize the receptor usage of this globally distributed pathogen, which may provide important insight into the virus’ evolutionary trajectory, interspecies transmissibility, and pathogenesis.

Results

The S1 Receptor Binding Subunit of the PDCoV S Protein Interacts with Host APN. In our search for PDCoV host receptor determinants, we screened known CoV receptors and detected binding of the S1 subunit of PDCoV S to porcine APN (pAPN). pAPN is a 963 amino acid-long type-II transmembrane glycoprotein, expressed as a homo-dimer on the cell surface. Transient expression of C-terminal HA-tagged pAPN in HeLa cells rendered these cells receptive to binding with Fc-tagged PDCoV S1 protein (Fig. L4 and SI Appendix, Fig. S1), similar to the S1 protein of TGEV that served as a positive control. Cell surface staining to pAPN-expressing cells was also observed with the isolated domain B of PDCoV S1 (S1B), indicating that this spike domain is responsible for pAPN binding (31, 32). HCoV-229E S1—known to bind human APN (hAPN) but not the porcine ortholog—did not bind to pAPN-expressing cells (43). Solid phase binding experiments confirmed the interaction between the PDCoV S1 and S1B with the pAPN ectodomain (Fig. LB); albeit TGEV S1 bound pAPN more efficiently, indicative of a higher binding affinity. These data demonstrate that the S1 receptor binding subunit of the PDCoV S protein directly interacts with the host APN transmembrane ectopeptidase with the interacting site residing in S1 domain B.

pAPN Is a Functional Entry Receptor for PDCoV. PDCoV can replicate in swine testis (ST) cells with supplemental trypsin (23). To determine the role of pAPN interaction in PDCoV entry, we used a mutant ST cell line lacking cell surface APN expression (ST-pAPNKO) that had been made previously using CRISPR/Cas9 genome editing (44). In addition, we generated an ST-pAPNKO cell line with reconstituted pAPN expression (ST-pAPNKO-pAPN). Integrity of the mutant cell lines was confirmed by sequencing. TGEV S1 cell surface staining (44) (SI Appendix, Fig. S2A), Western blotting (SI Appendix, Fig. S2B), and infection with TGEV control virus (Fig. 2A). Parental ST cells, ST-pAPNKO, and ST-pAPNKO-pAPN cells were inoculated with PDCoV at a multiplicity of infection (MOI) of 1 in the presence of trypsin and the percentage of infected cells was assessed after immunostaining. As revealed by flow cytometric and immune-fluorescence quantification, APN ablation in ST
cells reduced PDCoV infection by ∼75–90% relative to parental ST cells, whereas pAPN reconstitution in ST-APN^KO^ cells enhanced PDCoV infection beyond levels observed on parental ST cells, indicating that PDCoV uses pAPN as a receptor for infection (Fig. 2B and C). During infection, expression of CoV S glycoprotein at the plasma membrane can result in formation of multinucleated syncytia, which depends on and positively correlates with the local cell-surface receptor concentration. Following PDCoV infection, efficient syncytium formation was observed in ST and Vero cells overexpressing pAPN (ST-pAPN^KO^-pAPN and Vero-pAPN) but not in parental cells (SI Appendix, Fig. S3) [and, as earlier reported in wild-type ST cells (23)] that express low levels of APN (SI Appendix, Fig. S2A), suggesting that the APN glycoprotein can be employed by PDCoV S to mediate cell–cell fusion.

To further verify the role of pAPN in PDCoV infection, we used human HeLa and African green monkey Vero cells that were poorly susceptible to PDCoV infection, correlating with a lack of detectable APN expression in these cells (45–47). Mutant HeLa and Vero cell lines stably expressing pAPN were generated, and parental and mutant cell lines, as well as ST cells, were inoculated with PDCoV. pAPN expression rendered HeLa and Vero cells susceptible to PDCoV infection at levels similar to those observed on ST cells (Fig. 2D). In addition, we determined whether infection of parental and APN^KO^ ST cells as well as Vero cells stably expressing pAPN and its parental counterpart would support viral replication. To this end, we assessed the PDCoV growth kinetics through a multistep growth-curve experiment on these cell lines, supernatants of which were taken at set times and titrated on LLC-PK1 cells (SI Appendix, Fig. S4). The results indicate that the presence of APN enhances production of infectious PDCoV particles (∼100x increase in viral titers) and suggests that these cell lines are indeed permissive to productive PDCoV infection. Collectively, our data indicate that pAPN can act as an entry receptor for PDCoV infection.

**PDCoV Can Infect Cells of Galline and Human Origin.** The presumed origin of PDCoV in birds (13) and the well-known cross-species transmission potential of CoVs (6) led us to investigate the

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**Fig. 2.** Impact of APN expression on PDCoV infection. (A) PDCoV employs APN during ST cell entry. ST, ST-APN^KO^, and pAPN reconstituted knockout cells were inoculated with PDCoV or TGEV at a MOI of 1. Cells were fixed and permeabilized 16 hpi and stained using a PDCoV-specific antiserum or a TGEV-specific monoclonal antibody, respectively. Infection experiments were performed twice; representative pictures are shown. (Magnification: 300×.) (B) PDCoV infection levels on pAPN deficient and reconstituted ST cells were quantified by flow cytometry. Infection levels are shown normalized to parental ST cell infection. Experiments were repeated four times; averages are shown. (C) PDCoV and TGEV infection levels on pAPN-deficient and reconstituted ST cells quantified by counting virus-infected cells after immunofluorescent staining. Infection levels are shown relative to infection on the parental ST cells. Infection experiments were performed twice; a representative experiment is shown. (D) APN overexpression in HeLa and Vero cells potentiates PDCoV infection. HeLa and Vero cells stably expressing pAPN were generated by retroviral transduction. Cells were inoculated with PDCoV at a MOI = 1 (as titrated on ST cells) and infection levels were quantified at 16 hpi by flow cytometry. Experiments were performed two to five times. Average infection levels are shown relative to infection on the ST cells.
susceptibility of cell lines from species other than swine to PDCoV infection. To this end, we performed PDCoV infection experiments (MOI = 1.0) on cell lines derived from human and galline tissues. Remarkably, galline hepatoma (Leghorn male hepatoma, LMH) and fibroblast (DF-1) cells, as well as human hepatoma (Huh7) cells, appeared susceptible to PDCoV infection (Fig. 3). To determine whether APN contributes to PDCoV infection, we designed a flow-cytometric binding assay using interspecies APN chimeras. Human and feline APN (fAPN) were selected for chimera construction, as the wild-type proteins were observed to be bound strongly and undetectably by PDCoV S1, respectively, under flow-cytometric assay conditions, while these APN orthologs were also compatible with the use of S1 proteins of HCoV-229E (binds hAPN, no detectable binding to fAPN) and TGEV (can bind fAPN, no detectable binding to hAPN) as binding controls. Hence, we exchanged APN domains II or IV in hAPN by the corresponding fAPN domains and vice versa, and assessed the PDCoV S1 binding patterns to cell-surface wild-type and chimeric APN by flow-cytometry, alongside with TGEV and HCoV-229E S1 control proteins. Cell surface expression of the constructed APN chimeras was confirmed via staining by control CoV S1 proteins (Fig. 5B) or via detection of the C-terminally added HA-tag (SI Appendix, Fig. S7). Whereas PDCoV S1 binding to hAPN was lost upon exchange of domain II for the feline equivalent, PDCoV binding to hAPN was maintained when domain IV was swapped (Fig. 5B). Correspondingly, IAPN recognition by PDCoV S1 remained below the detection level after exchange with human domain IV, but was significantly increased upon swapping of domain II (Fig. 5B). Taken together, our data indicate that, like HCoV-229E, PDCoV engagement of APN critically depends on the catalytic domain II. However, it should be noted that, because IAPN is bound to low affinity by PDCoV S1 and can be used as an entry receptor by the virus (vide infra), no results can be drawn from any negative results obtained in this assay.

PDCoV S1 interacts with the Catalytic Domain of APN. Structural analyses of the APN ectodomain has revealed four independently folded domains, termed domains I through IV (48), which are highly conserved across animal species of the different vertebrate classes (Fig. 5A and SI Appendix, Fig. S6). Two of these domains are known to be targeted by the S1 subunits of other CoV species: HCoV-229E S1 engages APN via domain II, whereas TGEV S1 binds to domain IV (34, 49). To assess whether one of these two domains is also targeted by PDCoV, we designed a flow-cytometric binding assay using interspecies APN chimeras. Human and feline APN (IAPN) were selected for chimera construction, as the wild-type proteins were observed to be bound strongly and undetectably by PDCoV S1, respectively, under flow-cytometric assay conditions, while these APN orthologs were also compatible with the use of S1 proteins of HCoV-229E (binds hAPN, no detectable binding to fAPN) and TGEV (can bind fAPN, no detectable binding to hAPN) as binding controls. Hence, we exchanged APN domains II or IV in hAPN by the corresponding fAPN domains and vice versa, and assessed the PDCoV S1 binding patterns to cell-surface wild-type and chimeric APN by flow-cytometry, alongside with TGEV and HCoV-229E S1 control proteins. Cell surface expression of the constructed APN chimeras was confirmed via staining by control CoV S1 proteins (Fig. 5B) or via detection of the C-terminally added HA-tag (SI Appendix, Fig. S7). Whereas PDCoV S1 binding to hAPN was lost upon exchange of domain II for the feline equivalent, PDCoV binding to hAPN was maintained when domain IV was swapped (Fig. 5B). Correspondingly, IAPN recognition by PDCoV S1 remained below the detection level after exchange with human domain IV, but was significantly increased upon swapping of domain II (Fig. 5B). Taken together, our data indicate that, like HCoV-229E, PDCoV engagement of APN critically depends on the catalytic domain II. However, it should be noted that, because IAPN is bound to low affinity by PDCoV S1 and can be used as an entry receptor by the virus (vide infra), no results can be drawn from any negative results obtained in this assay.

PDCoV S1 Interacts with Galline and Human APN. We then examined whether the PDCoV S protein can bind cell surface-expressed hAPN and galline APN (gAPN). Again, HeLa cells were transfected with plasmids encoding either hAPN or gAPN, and subjected to CoV S1 cell surface staining. S1 proteins of TGEV and HCoV-229E were used for comparison. No TGEV S1 binding was assessed by immunostaining at 16 hpi. The results were confirmed in an independent experiment; pictures of a representative experiment are shown. (Magnification: Left, 200x; Right, 300x.)
Discussion
Receptor interaction is the first, essential step in virus infection of the host cell, and hence the key factor in defining a host range of viruses. We here demonstrate that PDCoV employs domain B of its S1 subunit to engage host APN as a receptor for cell entry. APN expression confers susceptibility of cells to infection by this pathogen. Infection with PDCoV was drastically reduced after knockout of APN in porcine or human cells, although no full block in infection was observed, indicating that APN-independent entry routes can be used by the virus in cell culture and permitting the possibility that PDCoV infection requires a coreceptor. The unusual receptor promiscuity of PDCoV and the ability of PDCoV to infect cells of avian and mammalian species warrant further investigation into the virus’ epidemiology and cross-species transmissibility.

APN is a multifunctional protein displaying enzymatic and other functions, including peptide processing, cholesterol uptake and chemotaxis to cell signaling, and cell adhesion (51). APN is widely distributed and highly conserved in amino acid sequence across species of the Animalia kingdom (SI Appendix, Fig. S6) and is expressed in a wide range of tissues, including the epithelial cells of kidneys (51), respiratory tract (52, 53), and gastrointestinal tract (54). Despite the wide distribution of APN in various tissues, PDCoV infection appears to be restricted to the swine enteric tract (55), indicating that factors other than receptor distribution play a role in CoV tissue tropism, such as differential distribution of cellular spike-activating proteases, which have been shown to play a decisive role in the CoV entry process (56). Remarkably, PDCoV shares its APN receptor with several members of the Alphacoronavirus genus, including TGEV and HCoV-229E (34, 35, 50), and thereby constitutes the second example of a CoV receptor that is shared across CoV genera, in addition to ACE2, which is recognized by the S proteins of alphacoronavirus HCoV-NL63 and betacoronavirus SARS-CoV (37, 38). While TGEV and other viruses of the Alphacoronavirus-I species bind APN via domain IV (49, 57), the alphacoronavirus HCoV-229E (58) and deltacoronavirus PDCoV engage domain II of their receptor. Despite involvement of the same receptor domain, the (proposed) receptor binding loops emanating from the S1 domains of the PDCoV and HCoV-229E S proteins lack sequence homology (SI Appendix, Fig. S9), similar to what has been observed for the receptor binding domains of SARS-CoV and HCoV-NL63 (59) and indicative of independent receptor acquisition during evolution. These observations suggest that APN has been independently selected as a receptor or at least two and likely three occasions during CoV evolution. The notable preferential employment of APN as a receptor by CoVs remains enigmatic but may stem from the abundant expression of this surface glycoprotein on epithelial cells of the intestinal (54) and respiratory tracts (52, 53), its inherent accessibility as a
peptidase, and the reported clustering of APN in specific (plasma)membrane microdomains with host cell transmembrane proteases that proteolytically prime CoV S proteins for membrane fusion (60). Considering the extensive CoV diversity in bats and their proposed role as the ultimate ancestral reservoir (61, 62), we posit that the proteinaceous receptor usage of CoVs is likely not limited to the four known cell surface glycoproteins (61, 62), we posit that the proteinaceous receptor usage of CoVs is likely not limited to the four known cell surface glycoproteins and that the repeated identification of these receptor molecules is merely a reflection of (effective) cross-species transmission compatibility with hosts of veterinary and medical importance.

Successful cross-species transmission depends foremost on the virus’ ability to bind and functionally use a receptor within an alternative host, causing the S protein to be the driver of CoV emergence. However, (changes in) non-S genes may code-terminate virus emergence in novel hosts (63). Our data indicate that PDCoV has access to cells of an exceptionally diverse range of species by binding to an interspecies conserved domain on APN. This resembles the situation for MERS-CoV, which recognizes its entry receptor DPP4 via a conserved binding site facilitating recurring zoonotic infections from its dromedary reservoir without the need for host receptor adaptation (64, 65). Selection of phylogenetically conserved receptors may provide viruses an evolutionary advantage by giving them leeway to explore alternative hosts, occasionally resulting in host switching and virus speciation (66). Considering its presumed avian origin, PDCoV’s functional engagement of orthologous receptors offers an attractive explanation for a mechanism that enabled a virus, ancestral to PDCoV, to breach the species barrier between birds and mammals. Structural studies on the PDCoV S1-APN complex may reveal the molecular basis for PDCoV’s remarkable receptor usage.

Our observations collectively reveal the multihost potential of PDCoV. A broad host range of PDCoV is also suggested by the reported susceptibility of germ-free calves to experimental PDCoV infection (67), as well as by the identification of a highly similar virus—at the time not yet recognized as a deltacoronavirus— in Chinese ferret badgers and Asian leopard cats at live-animal markets in southern China (68). As opposed to the repeated identification and isolation of PDCoV from swine, the incidental identification of viruses in both cat and badger with identical sequences seem to argue against a role for these animals as a potential reservoir (13). Whether these examples therefore represent spillover events from the pig reservoir, or have arisen from a yet unidentified (avian) host, remains to be seen.

The global distribution in swine of PDCoV with multihost potential is alarming from an epidemiological point of view. Pigs are the second largest livestock species (69) and acted as intermediate hosts for zoonotic viruses (70, 71), emphasizing the need for studying the zoonotic potential of the PDCoV and its surveillance in so far unappreciated potential reservoirs, including humans.

**Materials and Methods**

**Antibodies, Cells, and Viruses.** Polyclonal rabbit serum detecting HCoV-229E was kindly provided by Pierre J. Talbot, INRS-Institut Armand-Frappier.
Laval, QC, Canada (33); mouse anti-TGEV monoclonal antibody (ab20301) and mouse anti-HA epitope tag antibody (ab130275) were purchased from Abcam. The mouse monoclonal anti-PDCoV NP antigen antibody was purchased from Medgene Labs. Mouse monoclonal anti-dsRNA (J2) was purchased from Scicons. ST, African green monkey kidney (Vero-CCL81) cells, Madin-Darby canine kidney (MDCK) cells, human Huh7, DF-1, LLC-PK1, human embryonic kidney 293 cells stably expressing the SV40 large T antigen (HEK-293T), and derivatives of these cell lines were maintained in DMEM (Lonza BE12-741F) supplemented with 10% FBS (Bodinco). ST and Huh7 APN knockout cells have been described previously (44). LMH cells were maintained on plates coated with 0.1% gelatin in Waymouth’s media supplemented with 10% nonheat-inactivated FBS and 1 × penicillin/streptomycin.

Cells stably expressing pAPN (GenBank accession no. NP_999442.1) were made using the Moloney murine leukemia virus (MoMLV) transduction system (Clontech) by means of a pQCXIN retroviral vector encoding a pAPN cDNA sequence C-terminally extended with the HA-tag (YPYDVPDYA). Stably transfected pAPN cells were selected and maintained with G418 (PAA Laboratories).

Reference strain PDCoV virus was purchased from the US Department of Agriculture and was propagated and titrated on LLC-PK1 cells in DMEM with 1 μg/mL TPCK-treated trypsin (A370285, Sigma). PDCoV strain FD22 (passage 101) (23), which was propagated and titrated on ST cells, was used in infection experiments analyzed by flow cytometry. TGEV strain Purdue (GenBank accession no. ABG89335.1) was propagated and titrated on PDS porcine kidney cells. HCoV-229E was also propagated and titrated in DMEM supplemented with 1% of FBS but on human Huh7 cells.

Plasmid Design and Protein Expression. pCAGGS mammalian expression vectors encoding PDCoV S1 (isolate USA/Minnesota454/2014, residues 1–524; GB: AML40825.1) and its domain B (S1_298–425) C-terminally extended with the Fc domain of human or mouse IgG were generated as described before (44). Similarly, expression plasmids were made encoding Fc-tagged S1 subunits of TGEV (isolate Purdue, GenBank accession no. ABG89335.1, residues 1–785), HCoV-229E (GenBank accession no. NP_073551.1, residues 1–537), as well as constructs encoding human Fc-tagged soluble APN ectodomains (i.e., nonmembrane anchored) of swine (pAPN, GenBank accession no. XP_005653580.1), chicken (gAPN, GenBank accession no. ACZ95799.1; kindly provided by M. H. Verheije, Utrecht University, Utrecht, The Netherlands), human (hAPN, GenBank accession no. NP_001141.2), and cat (fAPN, NP_001009252.2) (44). Plasmids encoding the APN–hFc fusion proteins were polyethylenimine (PEI)-transfected into 60% confluent HEK-293T cells for 6 h, after which transfections were removed and medium was replaced with 293 SFM II-based expression medium (Gibco Life Technologies) and incubated at 37 °C in 5% CO2. Tissue culture supernatants were harvested 5–6 d posttransfection, and expressed proteins were purified using Protein A Sepharose beads (GE Healthcare) according to the manufacturer’s instruction. Purity and integrity of all purified recombinant proteins was checked by SDS/PAGE. Purified proteins were stored at 4 °C until further use.

Fig. 6. PDCoV can use APN orthologs of nonhost species as a receptor. Orthologous APN molecules facilitate PDCoV entry into cells. (Upper) HeLa cells mock-transfected or transfected with plasmids encoding pAPN, hAPN, gAPN, and fAPN, were inoculated with PDCoV, HCoV-229E, or TGEV at a MOI = 1 for 1 h. Cells were fixed and permeabilized 24 hpi and stained with virus specific antibodies (PDCoV, TGEV, and HCoV-229E) or a mouse anti-HA antibody (Abcam, ab130275) and corresponding Alexa594 conjugate. Infection experiments were performed twice; pictures of a representative experiment are shown. (Magnification: 300×.) (Lower) Quantification of PDCoV infection. Amount of cells and PDCoV+ cells were counted using five or more pictures for each infection condition, and expressed relative to infection in mock-transfected HeLa cells.
Immunofluorescence Assay. For immunofluorescence staining, cells were washed twice with PBS and fixed with 3% formaldehyde (Merck, 1040031000) in PBS, followed by nuclear permeabilization with 0.1% Triton X-100 (Sigma, 93426) in PBS for 10 min at room temperature. Fixed cells were blocked using 3% BSA (GE Healthcare Life Sciences) in PBS for 1 h followed by incubation with the primary antibody for 1 h in PBS with 1% BSA. Cells were washed three times and staining was completed by Alexa Fluor 488-conjugated goat α-rabbit antibody (A11008, Life Technologies) or Alexa Fluor 488-conjugated goat α-mouse antibody (A11001, Life Technologies). Nuclei were visualized using DAPI nuclear counterstaining (D-9542, Sigma). Pictures of immunofluorescent cells were captured using an EVOS FL Cell Imaging System (ThermoFisher Scientific) at 10x magnification or a Leica SPE-II confocal microscope (40x magnification). Relative infection was calculated by counting and averaging the percentage of infected cells in at least five microscopic fields per condition.

APN-Based Solid-Phase Binding Assay. The ability of the CoV S1- and S1-mFc fusion proteins to bind hFc-tagged soluble APN ectodomains was evaluated by means of an APN-based solid-phase binding assay. Per well, 100 μL of soluble APN-hFc (10 μg/mL, diluted in PBS) was coated in a 96-well MaxiSorp plates (Nunc) by overnight incubation at 4 °C. Nonspecific binding sites were calculated by counting and averaging the percentage of infected cells in at least 5 microscopic fields per condition.

PDCoV Infection Experiments. The cell culture conditions used to infect different cells with PDCoV were as follows: washing of cells with PBS two times, virus incubation for 2 h in fresh DMEM containing 0.5 μg/mL (in ST, HuH7, HeLa-R19, LMH, DF-1 cells and their derivatives) or in fresh DMEM containing 1 μg/mL (in Vero cells and its derivatives) of TPCK-treated trypsin (Sigma, 4370285). Cells were fixed and permeabilized at 12 h postinfection (hpi) and stained with virus-specific anti-sera.

Syncytium Formation Assay. Parental and mutant ST cells were inoculated with PDCoV at a MOI = 0.01 in DMEM containing 0.5 μg/mL trypsin at 37 °C. After 2 h, the inoculum was replaced by maintenance medium (without trypsin) and cells were further incubated at 37 °C to permit viral replication and subsequent protein cell surface accumulation. After 16 h, medium was replaced with DMEM or DMEM supplemented with 0.5 μg/mL trypsin for 3 h to induce proteolytic activation of exposed PDCoV S proteins and consequent cell–cell fusion, and subjected to immunostaining.

Flow Cytometric Quantification of CoV Infection. Cells that had been plated 1 d prior to were switched to enhanced MEM media (Gibco) containing 1% antibiotic-antimycotic (Gibco), 1% Hepes (Gibco), 1% (vol/vol) pancreatin (Sigma), and inoculated at a MOI = 1.0 for 1 h at 37 °C, after which inoculate was replaced by primary growth media containing 1% (vol/vol) pancreatin and cells were incubated overnight at 37 °C. Cells were fixed overnight in 100% ETOH at 4 °C, subsequently blocked for 1 h using 1x Power Bock Universal Blocking Reagent (Biogenex) at room temperature and stained overnight at 4 °C using a PDCoV-specific monoclonal antibody directed against the N protein (1:500; SDSU, mAb 55-197). TGEV staining was performed using a mix of two mAbs directed against the TGEV N protein (1:500; 25H7 and 14E3), which had been characterized previously (72–74). Cells were washed twice in PBS-T (PBS containing 0.1% Tween-20) before secondary antibody (1:400; goat α-mouse IgG Alexa488; Invitrogen) staining for 1 h at 37 °C. Cells were washed twice in PBS-T, resuspended in PBS, and analyzed using an Accuri C6 flow cytometer (BD Biosciences). A minimum of 20,000 events from three independent experiments was analyzed.

Flow Cytometric Analysis of Spike–Receptor Interactions. Human HeLa-R19 cells (ATCC) that had been plated 1 d prior to were mock-transfected or transfected with APN encoding plasmids using FuGene 6. Twenty-four hours after transfection, cells were released from tissue-culture dishes using 1 mM EDTA solution, washed, fixed in formaldehyde solution (4%), and blocked for 1 h. The APN conjugated polyclonal rabbit α-human immunoglobulins (1:2,000 dilution in PBS with 1% BSA; DAKO, P0260), and a colorimetric reaction was initiated through the addition of 100 μL/tube TMB (tetramethylbenzidine) Super Slow One Component HRP Microwell Substrate (Genway Biotech, GWB-7CD49) and stopped through addition of 12.5% H2SO4 (Merck). Optical density (OD) was subsequently measured at 450 nm with an ELISA reader (EL-808, BioTek). Background signal (α-mFc HRP-conjugate alone) was subtracted from the OD450 nm values. TGEV and HCoV-229E S1-mFc fusion proteins were used as binding controls.

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